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# THE BACTERIOLOGICAL EXAMINATION OF FOOD AND WATER

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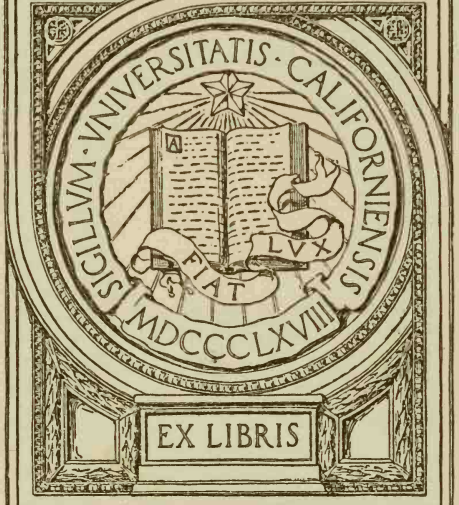

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# CAMBRIDGE PUBLIC HEALTH SERIES

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# THE BACTERIOLOGICAL EXAMINATION OF FOOD AND WATER

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# THE BACTERIOLOGICAL EXAMINATION OF FOOD AND WATER

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by

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# EDITORS' PREFACE

IN view of the increasing importance of the study of public hygiene and the recognition by doctors, teachers, administrators and members of Public Health and Hygiene Committees alike that the *salus populi* must rest, in part at least, upon a scientific basis, the Syndics of the Cambridge University Press have decided to publish a series of volumes dealing with the various subjects connected with Public Health.

The books included in the Series present in a useful and handy form the knowledge now available in many branches of the subject. They are written by experts, and the authors are occupied, or have been occupied, either in investigations connected with the various themes or in their application and administration. They include the latest scientific and practical information offered in a manner which is not too technical. The bibliographies contain references to the literature of each subject which will ensure their utility to the specialist.

It has been the desire of the editors to arrange that the books should appeal to various classes of readers: and it is hoped that they will be useful to the medical profession at home and abroad, to bacteriologists and laboratory students, to municipal engineers and architects, to medical officers of health and sanitary inspectors and to teachers and administrators.

Many of the volumes will contain material which will be suggestive and instructive to members of Public Health and Hygiene Committees; and it is intended that they shall seek to influence the large body of educated and intelligent public opinion interested in the problems of public health.

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# PREFACE

TEXT-BOOKS and Manuals upon Bacteriology are, for the most part, written by Bacteriologists whose investigations and routine work are in the field of Pathological Bacteriology. The descriptions in these text-books of methods suitable for the examination of morbid human materials and for the study of the pathogenic bacteria leave little to be desired on the score of either lucidity or completeness.

In marked contrast is the inadequate treatment given to the bacteriological examination of water, air, foods and the like. The description of their examination is either relegated to a few pages at the end of the volume, where their treatment is both scanty and incomplete, or, not infrequently, is omitted altogether. This branch of bacteriology is of immense practical importance and justifies a more extended treatment.

The aim of this volume is to remedy this defect and to make available a practical manual dealing not only with the examination of these substances but also with the deductions to be drawn from the bacteriological data obtained from their examination. Much of the available information is only at present to be found in original papers not always readily accessible.

The methods selected are, in general, those which I have found to be of practical value and proved utility, while special attention has been given to the difficult matter of the deductions

to be drawn from bacteriological examinations, and how far they may be used as a basis of administrative action.

Figures 1 and 16 and figures 8, 9, 10, 11 and 13 are taken from my books *The Bacteriological Examination of Water Supplies* and *Milk and the Public Health* respectively, and I am greatly indebted to Mr H. K. Lewis and Messrs Macmillan and Co. for their permission to use these illustrations.

I have also to thank Messrs W. B. Saunders and Co. for figure 3 (from Eyre's *Bacteriological Technique*), Messrs Longmans, Green and Co. for figure 12 (from Curtis's *Essentials of Practical Bacteriology*), Messrs Baird and Tatlock for figures 5 and 15, and Messrs A. Gallenkamp and Co. for figure 7.

WILLIAM G. SAVAGE.

WESTON-SUPER-MARE.
*February* 1914.

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# CHAPTER I

## GENERAL CONSIDERATIONS

Although water, food, air, etc. are not all examined bacteriologically with the same objects, broadly there is a similarity of procedure and certain general considerations may be conveniently considered together, in this way avoiding repetition in the chapters dealing with the examination of the different substances.

Bacteriological examinations of food and other substances are undertaken essentially for one or other, or for all, of the following purposes.

(*a*) To examine for the presence of definite disease-producing organisms. This is done either with the object of detecting their presence or, on the negative side, to judge by the failure to find them, whether they are absent or, if at one time present, have been eliminated.

(*b*) To *measure* the extent to which the substance under examination has been polluted by material derived from undesirable or harmful sources.

(*c*) To assess the value and completeness of any purification processes to which the substance under examination has been subjected.

The examination for specific disease-producing organisms is a procedure of great importance but is of limited applicability and practical utility in connection with the examination of water and foods. The detection of pathogenic bacteria in the human or animal body and in many of their excretions is, with modern

methods, often a procedure of no great trouble or complexity. On the other hand their isolation from water, food, air, etc. is frequently a matter of the utmost difficulty for reasons which readily present themselves. In these substances any pathogenic bacteria are frequently present in but small numbers compared with the total number of bacteria present, while these competing bacilli, by growing more readily and abundantly in the media used for the isolation of the pathogenic bacteria, may materially enhance the difficulties of isolation. Further, there is always the possibility that any pathogenic bacteria originally present may have been completely eliminated by the time attention is called to the need for bacteriological examination. These pathogenic bacteria in water, soil or even in milk are, or may be, in an environment either in itself unsuitable to their multiplication and sustained life or in one relatively unsuitable compared to that enjoyed by the horde of naturally present, or added, competing bacilli. This may cause their actual or relative disappearance when the food or water is submitted to bacteriological examination. For these reasons the isolation of specific pathogenic bacteria plays but a small part in this class of bacteriological analysis.

The second object of a bacteriological examination—to measure undesirable bacterial contamination—is by far the most valuable part of the bacteriological examination of the materials under consideration. By modern methods it is possible not only to say whether such pollution exists but, with considerable approach to accuracy, to determine the extent of such pollution and to furnish data as to the degree of its harmfulness.

Although the substances to be examined are various while the sources of pollution are many and not all of the same character there is for most of them a broad relationship as to the nature of the pollution to be detected and measured. For example, for water supplies bacteriological analysis is mainly concerned with the detection and estimation of excretal and sewage pollution. For shellfish the degree of sewage pollution is again the object of the examination. For milk, apart from the detection of pathogenic bacteria, the examination is chiefly

to study bacterial contamination mainly from cow manure or excreta-laden dust. Although the objects of the bacteriological examination of air are somewhat different it is in general true to state that excreta (human and animal) and sewage are the most important forms of pollution which the Public Health Bacteriologist has to detect and estimate in connection with his work. To do this the bacteriologist has to determine the organisms which are prevalent in sewage and excreta and which can be used by him to estimate the extent of the contamination by these substances.

These *indicator* organisms are of great importance. Those which have been most widely used for this purpose are the *B. coli* group, streptococci, and *B. enteritidis sporogenes* and closely allied anaërobic bacteria. It will be convenient to consider first the general and special characters of these groups of bacteria and subsequently to deal with the grounds on which they have been selected as suitable to measure excretal and sewage contamination.

### *B. coli group.*

A study of the characters and distribution of this group is of the utmost importance to the bacteriologist who has to deal with the examination of water, milk and other foods. Difficulty is met with at the outset in defining the characters of the group and it is easy to err on the one hand by adopting too comprehensive a definition, including organisms essentially different and with different distributions in nature, and on the other, by limiting the group by too narrow a characterisation, excluding organisms of material importance and significance.

All the members of the group are small non-sporing bacilli, decolorised by Gram's method of staining, which grow well at both 37° C. and 20° C., which ferment both glucose and lactose, with the production of acid and gas, and which fail to liquefy gelatine (within a reasonable period). Motility is present or can be demonstrated by the use of proper media and technique but it is not always exhibited and its absence in ordinary examinations cannot be taken as excluding organisms from the group.

Such a characterisation is a very wide one and will include a large number of organisms which certainly are not all alike, while many of them will not be identical with the *B. coli communis* of Escherich. On the other hand these characters will separate the group from the Proteus, Gaertner, Dysentery groups, etc.

All the members of this large group have not an equal significance as indicators of excretal contamination so that it is important to carry the classification further.

The cultural characters which have been employed for the differentiation of the bacilli of this group are very numerous but in regard to a number of them experience has shown that no differentiation of value is likely to result from their employment and they may be disregarded. Of the differentiation tests still considered by many bacteriologists to be of value the following are the most important :—Production of indol, growth in litmus milk, characters of the growth on gelatine slope, production of fluorescence in neutral red media, Vosges and Proskauer's reaction, and the fermentation of various sugars and alcohols.

The value of these tests can be best gauged not only by their ability to adequately differentiate the group as a whole but also by the light they throw upon the distribution of the different members of the group. If by their use differences of distribution are made manifest, so that the finding of the one variety indicates one kind of contamination while the presence of another variety points to a different source of contamination it is obvious that the tests which enable this to be done are of the utmost value.

Excreta—human and animal—is the most important form of pollution for which this group is used as a test and the value of these differentiating reactions must largely depend upon how far they satisfactorily differentiate the strains found in excreta from those which have not recently been derived from the animal intestine.

The characters of *B. coli* as found in excreta have been investigated by several workers of whom the following may be mentioned.

Houston[1] studied the characters of 101 Coli-like organisms

[1] *Local Government Board, Medical Officer's Report*, 1902–3, p. 511.

isolated from normal stools of healthy persons. Of these 8 did not ferment lactose although they fermented glucose. Of the remaining 93, 72 were completely positive as regards fermentation of glucose and lactose, positive neutral red reaction, production of indol, acid and clot in litmus milk and reduction of nitrates, 12 were non-motile, otherwise typical, 2 produced no indol as their only abnormality, 2 failed to give the neutral red reaction, 4 were typical except that they did not clot milk. That is leaving out motility as a distinguishing character 84 per cent. were completely positive in their characters.

Of these organisms about half fermented dulcite while considerably less than half fermented saccharose.

Houston[1] obtained very similar results with 60 *B. coli* organisms isolated from fresh cow-dung. 95 per cent. gave acid or clot in milk, 96·7 per cent. gave indol, 96·7 per cent. acid and gas in lactose media, 98·3 per cent. a positive neutral red reaction.

53 out of the 60 (88 per cent.) were quite typical as regards all these tests.

MacConkey[2] records the examination of 178 samples of human faeces and 131 samples of animal excreta, derived from the horse, calf, goat and pig. Ignoring for the moment most of the sugar-alcohol tests which were also employed, and excluding a few yellow liquefying organisms certainly not members of the group the results obtained may be tabulated as follows:—

| | Strains isolated | | Percentages | |
|---|---|---|---|---|
| | Human | Animal | Human | Animal |
| Milk clotted ... ... | 178 | 126 | 100 | 100 |
| Indol formed ... ... | 164 | 122 | 92 | 96·8 |
| Gelatine not liquefied ... | 175 | 124 | 98·2 | 98·4 |
| Saccharose fermented | 76 | 56 | 42 | 44·4 |
| ,, not fermented | 103 | 70 | 58 | 55·6 |

All the organisms fermented both glucose and lactose.

MacConkey strongly advocates the use of fermentation tests including lactose, saccharose, dulcite, adonite, inulin, inosit and mannite, and the abandonment of many of the old tests. In

[1] *Ibid.* 1904–5, p. 358.

[2] *Journ. of Hygiene*, 1909, IX, p. 86.

this way he has differentiated the strains isolated from different sources into a large number of different varieties. His lines of classification have been followed by other workers. The value of their employment must depend upon the light they shed upon the differential origin of the different strains. For example, to demonstrate that the fermentation of saccharose test is of value it has to be shown that organisms which ferment this sugar have a greater or a lesser significance than those which do not ferment saccharose. If all *B. coli* in excreta, or even a preponderance of them, fermented saccharose, then the presence of a *B. coli* in water or food which was without this property would have but little significance as an indicator of excreta. Nothing of the sort, however, has as yet been established, and the same may be said for the other sugar-alcohol tests, and interesting as these tests are and valuable for research work, it cannot be said that their employment for *routine* work adds information of material value.

The testing of the pathogenicity of isolated strains of *B. coli* has not proved of any practical utility for public health work.

For routine practical work it is important to have some definite and easily carried out system for recording *B. coli* organisms and noting how far they conform to the type most frequently met with in sewage. In 1905 the writer suggested[1] that the expression *excretal B. coli* should be used for organisms giving all the following characters:

A short rounded bacillus.

Translucent non-corrugated growth on gelatin slope.

Non-liquefaction of gelatin (two weeks).

Acid production in litmus milk with coagulation (within two weeks).

Fermentation of lactose with production of acid and gas.

„ glucose „ „ „

Neutral red reaction (in glucose media).

Production of indol in peptone water.

Since practically all organisms which ferment lactose also ferment glucose the fermentation of glucose may be omitted,

[1] *Lancet*, Feb. 4, 1905.

while the neutral red reaction is not of much assistance: otherwise these tests are all valuable and satisfactory.

Used in this way the term *excretal B. coli* would imply without further description that all these characters were present. These characters are certainly those possessed by the vast majority of *B. coli* isolated from excreta and the term is a much more convenient one than *typical* since to the latter so many different interpretations have been given. If any characters are negative, the fact can be mentioned in brackets. Thus an organism having all these attributes, except that it gives no indol reaction, can be readily written *excretal B. coli* (indol –).

### *Streptococcus group.*

The term streptococcus being only a description of a morphological type, must obviously include a number of different organisms, although Marmorek and other investigators hold that all human streptococci are identical. Many different kinds of streptococci have been described and much discussion has taken place as to how far the described varieties are separate species or mere variants of a common type. The methods in use to classify streptococci are very numerous, but it cannot be said that their differentiation by any method is completely satisfactory nor is the basis of classification uniform for different workers.

Some investigators rely solely upon the characters most used in earlier bacteriological investigations, such as morphology, pigment-production, characters of the gelatine plate colonies, growth in broth, milk, potato, etc. Other workers place in addition to, or in substitution for, these tests, great reliance upon pathogenicity, agglutination tests, or the haemolytic tests introduced by Schottmüller. A further group of workers base their differentiating characters in large part upon the ability of streptococci to produce acid in certain sugars or alcohols.

It cannot be said that any one series of tests is satisfactory or sufficient, but speaking generally the sugar-alcohol tests, together with morphology, growth in broth and milk, and pathogenicity are in the writer's experience of most utility.

It should, however, be stated that several investigators have questioned the reliability and value of these sugar-alcohol tests and have not found them to be sufficiently stable.

These tests were introduced by Gordon[1] and included the following nine tests: Litmus milk for clot (three days at 37° C.), reduction of neutral red broth during anaërobic incubation for two days at 37° C., production of acid in three days at 37° C. in slightly alkaline broth containing respectively 1 per cent. of saccharose or lactose, raffinose, inulin, salicin, coniferin, or mannite. With the exception of the use of coniferin these tests have been extensively employed.

Houston in 1902 studied the biological characters of 300 human faecal streptococci[2], in 1903 the characters of 100 streptococci from cow-dung[3], and in 1908 he investigated the characters of 100 streptococci isolated from lumps of faeces taken at a sewage outfall works[4].

These 500 streptococci showed the following percentage positive reactions:

| Test | Human Faeces | Cow-dung | "Sewage" |
|---|---|---|---|
| Salicin ... ... ... | 92·7 | 93 | 97 |
| Saccharose ... ... | 86·3 | 89 | 49 |
| Lactose ... ... ... | 76·3 | 85 | 100 |
| Litmus milk ... ... | 61·7 | 73 | 100 |
| Neutral red broth ... | 39·3 | 0 | — |
| Raffinose ... ... ... | 32 | 74 | 100 |
| Mannite ... ... ... | 24·3 | 0 | 4 |
| Inulin ... ... ... | 4·7 | 13 | — |

The biological characters of streptococci from excreta have also been studied by Winslow and Palmer[5]. They examined 302 streptococci, 116 from human, 100 from horse, 86 from cow excreta. They employed similar methods but the amount of acid was in each case estimated by titration, using phenolphthalein as indicator. Each culture was incubated 72 hours at

[1] *Local Government Board, Medical Officer's Report*, 1903–4, p. 388.
[2] *Ibid.* 1903–4, p. 484.
[3] *Ibid.* 1904–5, p. 326.
[4] *Metropolitan Water Board, Fifth Research Report.*
[5] *Journ. of Inf. Diseases*, 1910, vol. VII, p. 1.

37° C. Only glucose, lactose, raffinose and mannite were employed. Their percentages of positive results were as follows:

| | Human | Equine | Bovine |
|---|---|---|---|
| Glucose ... ... | 89 | 84 | 65 |
| Lactose ... ... | 62 | 8 | 52 |
| Raffinose ... | 6 | 4 | 28 |
| Mannite ... ... | 28 | 2 | 6 |

Streptococci from sources other than excreta tested in the same way do not show differences sufficiently distinctive to enable the excretal origin of any streptococcus to be ascertained from an examination of its biological properties.

For the examination of water, soil, etc. the differentiation of the isolated streptococci has not up to the present proved to be of any immediate practical value.

A classification of streptococci on this basis has on the other hand proved to be of value in differentiating certain human streptococci and for the identification of streptococci in air. The classification adopted is discussed in Chapter IX.

### *B. enteritidis sporogenes.*

This bacillus was isolated in October, 1895, by Klein from the intestinal discharges of patients at St Bartholomew's Hospital suffering from an epidemic of diarrhœa. It was also isolated from the milk supplied to these patients.

It is a strictly anaërobic organism with fairly definite characters, of which the most important are the following: A fairly large bacillus 1·6 to 4·8 $\mu$ long and about 0·8 $\mu$ thick. Motile, and stains by Gram's method. It readily forms spores, which are usually present near the ends of the rods.

Under anaërobic conditions it grows well in milk, glucose agar, and upon blood serum and other media. Milk cultures are especially characteristic, and this medium is usually used for its isolation. The milk is coagulated and separated into a stringy material (coagulated casein) and a clear or somewhat turbid whey, a scattered layer of cream remaining on the top, which is broken up by the gas which is readily liberated by shaking the tube. The whey is acid, and the tube-contents smell of butyric acid. The whey examined microscopically shows the bacilli.

The above cultural and microscopic characters are very similar to those of *B. butyricus*, and probably some other organisms, so that it is necessary to make inoculation experiments if it is required to accurately differentiate these bacilli, *B. enteritidis sporogenes* being highly pathogenic, and *B. butyricus* not pathogenic.

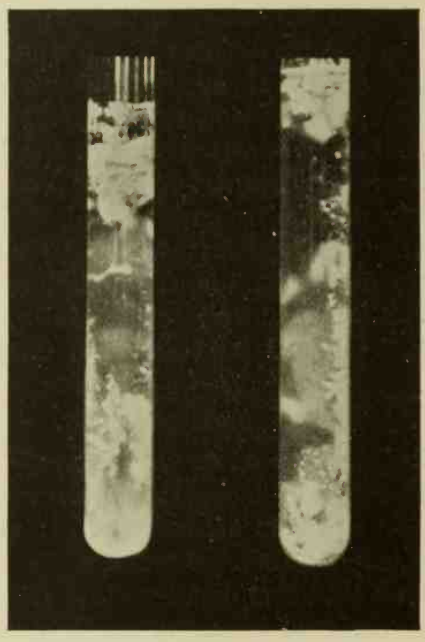

Fig. 1. *Bacillus enteritidis sporogenes.* Typical milk cultures, after 24 hours' incubation at 37° C.
(From photographs kindly lent by Dr Klein.)

Klein recommends that 1 c.c. of the whey from the milk culture be injected subcutaneously into the groin of a guinea-pig. The animal will usually be found dead within twenty-four hours. On making a post-mortem examination the hairs near the site of inoculation are readily stripped off, the skin underneath being green and gangrenous. Beneath the gangrenous area extensive sloughing of the subcutaneous tissue has taken place, and a considerable blood-stained, evil-smelling fluid is present, containing numerous bacilli with the characters given above. The bacilli

never show the smooth unseptate filaments characteristic of the bacillus of malignant œdema. If cultures of diminished virulence are used the pathogenic effects are somewhat modified. The virulence of this organism is subject to considerable variation, to some extent dependent upon its source.

The changes in milk cultures and the demonstration of the pathogenicity of the whey, along with the post-mortem appearances described, are quite sufficient for the identification of this organism.

Since *B. butyricus* and the other culturally nearly identical organisms have a very similar distribution in Nature, it is not usually considered necessary to carry out the pathogenicity test in every instance. The diagnosis is then based upon the characteristic "enteritidis change" in the milk.

*Bacterial indicators of excretal and sewage contamination.*

These three groups—*B. coli*, streptococci and *B. enteritidis sporogenes*—are very extensively employed to estimate the extent of excretal or sewage contamination and it is of great importance to consider critically the value of these determinations for this purpose.

The conditions of a perfect bacterial indicator are fairly definite and obvious. They are:

1. It should be abundant in the substances, for which its presence serves as an indicator.

2. It should be absent, or at least relatively absent, from all other sources.

3. It should be easily isolated and numerically estimated.

4. Its characteristics should be definite and not liable to variation, whereby its distinctive characters might be impaired.

All three groups are extremely abundant in both human and animal excreta and in sewage.

As regards human excreta *B. coli* group organisms are present to the extent of 100 million to 1000 million or more per gramme, streptococci are usually equally numerous, while about 1 million to 10 million *B. enteritidis sporogenes* are present per gramme.

These organisms also occur in immense numbers in the intestines of all the domestic animals and apparently of all mammals. The following table shows some results obtained by the writer with quite fresh specimens of animal excreta.

| Source | *B. coli* | Streptococci. | *B. enteritidis sporogenes* Spores |
|---|---|---|---|
| | Approximate number per Gramme of Excreta | | |
| Horse No. 1 | over 1 million | $\frac{1}{10}$ to 1 million | 10 to 100 |
| ,, No. 2 | $\frac{1}{10}$ to 1 million | over 1 million | 100 to 1000 |
| ,, No. 3 | 1,000 to 10,000 | over 1 million | 100 to 1000 |
| Cow No. 1 | $\frac{1}{10}$ to 1 million | 10,000 to 100,000 | 100 to 1000 |
| ,, No. 2 | 10,000 to 100,000 | $\frac{1}{10}$ to 1 million | 10 to 100 |
| ,, No. 3 | 1 to 10 millions | over 10 millions | 10 to 100 |
| ,, No. 4 | 1 to 10 millions | $\frac{1}{10}$ to 1 million | 100 to 1000 |
| Pig No. 1 | over 100 millions | absent | $\frac{1}{10}$ to 1 million |
| ,, No. 2 | 10 to 100 millions | absent | 10,000 to 100,000 |
| ,, No. 3 | 70 millions | absent | 1000 to 10,000 |
| Sheep No. 1 | 10 to 100 millions | 1 to 10 millions | 10 to 100 |
| ,, No. 2 | 10 to 100 millions | 10 to 100 millions | 10 to 100 |

The distribution of streptococci and *B. enteritidis sporogenes* in animal excreta has not been so fully worked out, but as regards *B. coli* this group has been found to be abundant in the excreta of many birds and fishes. Their occurrence in birds and fishes is of importance in connection with the bacterial content of upland waters which may be contaminated with these organisms from this source.

Houston has examined the excrement of gulls and found *B. coli* in enormous numbers. Streptococci and spores of *B. enteritidis sporogenes* were present in the excreta of two only (out of eight) of the gulls and in only small numbers. Eyre has also found *B. coli* abundant in a gull and a number of other birds.

Eyre and Johnson have independently examined a number of fishes, the former from the sea, the latter from river waters and both found *B. coli* to be present in the intestinal contents.

All three groups of organisms are abundant in crude sewage. The actual numbers found will of course vary greatly with the strength of the sewage, but the data given by Houston give average figures. These are *B. coli* and allied forms about

100,000 per c.c., streptococci 1000—10,000 or more per c.c. and spores of *B. enteritidis sporogenes* 100 to 1000 per c.c.

The second condition is that these indicator organisms should be absent or relatively absent from sources other than those for which they are to serve as an index. A great deal of work has been carried out upon the distribution of these organisms in saprophytic surroundings and while the results are not in complete accord the work done has established certain general facts in favour of the use of these organisms (and more particularly the *B. coli* group) as a satisfactory measure of excretal or sewage contamination.

Dealing first with *B. coli* in soil the general facts set out in Chapter IV show that members of this group are only present when the soil has been contaminated with excrementitious matters and that virgin soil and soil not manured are free from these organisms. Further it has been shown that these organisms gradually die out in soil.

Members of the *B. coli* group have also been found to be present on wheat, rye and other grain and a number of investigations in this direction have been made. The results, considered broadly, show that when the grain is carefully collected directly from the fields no *B. coli* are present, but that if the examinations are made after ordinary storage members of this group are frequently found in small numbers. It is to be anticipated that stored grain will often show *B. coli* on examination in view of the fact that these organisms are abundant in the excreta of rats and mice, animals generally numerous in grain ships and storage places.

In quite pure water, pure air, etc. *B. coli* group organisms do not occur.

Briefly stated, we have, in the *B. coli* group, organisms which are extremely abundant in excreta and sewage, but which do not occur in air, water, soil or other substances unless they have been in contact with excrementitious matter, while they do not multiply to any extent under ordinary natural conditions, outside the animal body.

As regards the saprophytic distribution of streptococci there is no evidence that streptococci have any true home, under

natural conditions, apart from the animal body. As Andrewes[1] points out, "They are certainly abundant in the air of London, but these common air-streptococci are, as I have elsewhere shown, identical with the forms most abundant in the horse-dung which is amongst the chief constituents of London street dust. They occur, too, in water, but in proportion to its contamination with sewage, or at least, with animal excreta. Much the same is true of earth and soil. In all these situations streptococci may, indeed, be found, but always, it would seem, as the result of pollution with organic matter."

Their presence in contaminated and absence from pure soil is shown in Chapter IV.

Outside the animal body they may survive for considerable periods but do not thrive. The evidence as to the duration of viability and vitality outside the animal body is somewhat conflicting, but in general it would appear that the majority are delicate organisms and rapidly die out, but that a small number of hardy strains may persist for very long periods.

In relation to their viability in water one of the most recent investigations is that of Houston[2], who studied the viability of 100 streptococcus strains, isolated from lumps of excreta in sewage, in Thames river water sterilized by passing through a Pasteur filter. About 100,000 of each of the 100 varieties were added separately to 100 tubes, each containing 10 c.c. of the filtered water. Cultures (1 c.c. of water) were made from them each week. The tubes were kept in a dark cupboard at a temperature varying from 8° to 16° C. The following is a summary of the results:

| | | | |
|---|---|---|---|
| 3 | per cent. | died in | 1 week. |
| 11 | „ | „ | 2 weeks. |
| 32 | „ | „ | 3 „ |
| 38 | „ | „ | 4 „ |
| 8 | „ | „ | 5 „ |
| 4 | „ | „ | 6 „ |
| 2 | „ | „ | 9 „ |
| 1 | „ | still alive in | 9 weeks. |

(one tube became contaminated.)

[1] *Lancet*, 1906, II, Nov. 24 (Horace Dobell Lecture).

[2] *Metropolitan Water Board, Fifth Research Report.*

Our knowledge as to the distribution and viability of the different varieties of streptococci is very incomplete.

As regards the distribution of *B. enteritidis sporogenes* under saprophytic surroundings the investigations made have usually not accurately differentiated this organism from very closely allied forms, and the results which have been obtained for the most part refer to the distribution of the spores of the organisms which cause the so-called "enteritidis change" in milk. This change is, however, usually due to *B. enteritidis sporogenes*, and for practical purposes the results obtained may be taken as showing the distribution of this bacillus.

The spores of this bacillus are present in soil and, while they are more abundant in polluted than in virgin soils, they are present to some extent in soils not recently contaminated (see Chapter IV).

Klein and Houston found virulent *B. enteritidis sporogenes* spores present in most of the samples of grain (wheat, oats, rice, oatmeal and wheat-flour) examined. One gramme had usually to be examined before positive results were obtained. Balfour Stewart also found the spores prevalent in grain.

In considering the value of this organism as an excretal indicator, it must be remembered that it is a spore-bearing bacillus and that its spores are very resistant. Animal excretal pollution is so widespread that it is not a matter of surprise that such a highly resistant organism should be widely distributed in nature. The available evidence shows that it is absent, or relatively absent, from sources which have never been contaminated, but that it is fairly prevalent in sources the pollution of which had taken place possibly at a long antecedent period. These considerations obviously place a considerable limit to its usefulness.

The other conditions of suitable bacterial indicators refer to the readiness with which they can be isolated, estimated and accurately defined. All three groups, although they possess drawbacks, fulfil these conditions. From this point of view the streptococci are the least satisfactory, and really satisfactory methods for their isolation and differentiation are greatly needed. Thanks to the numerous investigations which have

been made and particularly to the introduction by MacConkey of the use of bile salt, the isolation and identification of *B. coli* has now been placed on a very satisfactory footing.

In general it may be said that the *B. coli* group is by far the most reliable indicator of excretal pollution which we possess and its use is equally applicable for water, shellfish, soil and other substances. The other two indicators are chiefly of value for confirmatory purposes.

These bacteria not only indicate excretal contamination, but they can be used to measure its extent by means of careful numerical estimation. This is a matter of extreme importance, since excretal pollution is so widespread that to some degree evidence of it must also be widespread, and measurement, not mere detection, is required.

## CHAPTER II

### GENERAL METHODS FOR THE ISOLATION AND IDENTIFICATION OF INDICATOR ORGANISMS

While for the isolation and identification of indicator organisms considerable variations are required in detail, according to the material submitted for examination, the general procedures are identical whatever the source. It is therefore convenient and saves repetition to discuss them together in a single chapter.

The methods used must be capable not only of detecting the indicator organism but must be able to estimate the number present in a given sample. In this they differ from procedures used for the isolation of pathogenic bacilli such as *B. typhosus* or *Sp. cholerae*, since for these detection is all that really matters.

In the previous chapter it has been explained that the three most useful groups of organisms for the estimation of excretal contamination are *B. coli* and allied organisms, streptococci and *B. enteritidis sporogenes*.

The examination and isolation of these organisms involve three considerations:

(*a*) A preliminary determination of their probable presence.

(*b*) Their isolation in pure culture.

(*c*) The application of determining tests.

Sometimes *a* and *b* are combined.

### *Bacillus coli Group.*

To prevent the separate examination and plating of all the different quantities of water, milk, or other substance selected for examination it is necessary to have some means by which those amounts which contain *B. coli* organisms can, with some measure of probability, be distinguished from those which are free from this organism. Authorities are not in complete agreement as to the respective value of glucose and lactose fermentation, but the great majority of workers select the fermentation of lactose as the essential primary means of differentiation since this character is a convenient and satisfactory one to employ.

By the employment of some agent such as litmus or neutral red in conjunction with lactose to indicate when the acid fermentation of this sugar has taken place, or by the use of double tubes so that the formation of gas can be seen, it is possible to differentiate between liquid media tubes (or individual colonies when solid media are employed) which respectively do not or which do contain lactose-fermenting bacilli. Such means of differentiation are of immense importance in routine work as they enable tubes or plates not containing lactose-fermenting bacilli to be at once discarded. All modern methods make use of some such means of differentiation.

For liquid media the fermentation of lactose with production of *gas* as well as acid is by far the best means of differentiation, with solid media the production of *acid* alone has to be relied upon.

In regard to the comparative value of liquid and solid media for this primary differentiation there are considerable differences of opinion.

The use of tubes of liquid media involves two possibilities of considerable error. One is that the estimations obtained only give very widely spaced results. For example, with water samples, 0·1, 1, 10 and 100 c.c. are the amounts usually selected for examination, and according to the positive findings the number of *B. coli* organisms will be 10,000, 1000, 100, 10, etc. to the litre. There is obviously a considerable spacing between these results. This wide spacing can be largely obviated by the use of more tubes but this greatly increases the labour and therefore in practice this method of diminishing the possible error is limited. The other objection is that bacteria are by no means uniformly distributed and if the bacilli under examination happen to be present in the smaller amounts used for examination, misleading results may be obtained and a much greater prevalence recorded than is warranted by the facts.

This second objection also largely applies to solid media enumeration methods, while these methods have several other decided drawbacks of their own. One of the chief of them is that they enable small quantities (*i.e.* 0·1 to 1 c.c.) only of the material under examination to be dealt with. It is not very convenient to concentrate the bacteria into a smaller bulk, by filtration or other method, so that this method is not suitable for many substances.

A further decided objection to their use is that they give unreliable colony differentiation. Theoretically the medium used should clearly differentiate the *B. coli* group colonies from the rest of the bacteria by their being coloured red or otherwise distinguished; in practice there are many intermediate colonies which it is not possible to say, without further cultural differentiation, whether they belong to the *B. coli* group or not. In particular if the plates are crowded true *B. coli* group bacilli will frequently not develope into characteristic colonies until after several days so that there is great danger of their being unenumerated as *B. coli* colonies. The presence of these indeterminate colonies seriously reduces the accuracy and value of the direct plating method and makes the results obtained dependent to some extent upon the recorder. From extended comparative work with the examination of milk and other substances the

writer decidedly prefers liquid media for primary enumeration. It should, however, be said that a number of authorities prefer the solid plate method[1].

A second object to be aimed at in the detection of *B. coli* and other indicator organisms is to use means whereby the growth of these organisms is favoured while that of other bacteria is retarded. A simple way of doing this is to employ a temperature which selectively favours the growth of the bacilli required. Vincent suggested a temperature of 42° C., MacConkey used at first a temperature of 43° C., while in Eijkmann's method a temperature of 46° C. was employed. *B. coli* organisms grow well at 37° C. and the advantage of these higher temperatures is not commensurate with the trouble of using special incubators.

Growth under anaërobic conditions was for similar reasons advocated by Pakes, but the advantages are not great enough to balance the extra trouble and other methods are better.

Selective growth may also be favoured by the addition of certain chemicals. Studies along this line have been very valuable and fruitful. Phenol was at one time extensively employed but is now largely given up as it does not satisfactorily differentiate.

We owe to MacConkey the most valuable substance for selective differentiation in the bile salts, usually employed as sodium taurocholate. This substance is a satisfactory inhibitory substance, while in the amounts used it does not interfere with the growth of *B. coli*.

The inhibitory action of certain colouring media, such as crystal violet, have also been successfully employed.

Of all these agents it may be said that the use of lactose bile salt broth in double tubes is the most valuable when the liquid enumeration method is employed, while for solid media any of those mentioned in the next stage may be employed. The composition of these media is given in the Appendix.

The second stage of the examination consists in the isolation of the bacillus in pure culture. If solid media were employed

[1] For a good discussion of this question and in favour of solid media see Gärtner, "Das Bacterium coli als Indikator für fäkale Verunreinigung eines Wassers," *Zeit. f. Hyg.* 1910, vol. 67, p. 55.

for the first stage this is already carried out, but if liquid differentiating tubes are employed the bacillus has to be isolated from the tubes selected.

The principles enunciated above apply equally here and it is a great advantage to employ media containing both a differentiating agent and a substance which retards the growth of bacteria other than those to be isolated.

On these grounds the use of ordinary gelatine and agar have been given up and coloured media employed.

Of such media lactose bile salt neutral red agar (L.B.A.), nutrose agar (Drigalski-Conradi agar), and fuchsin agar may be specially mentioned.

The ingenious aesculin agar may also be mentioned here. This contains the glucoside aesculin and ferric citrate. The action of *B. coli* causes the aesculin to combine with the iron citrate and form a dark brown salt, the *B. coli* colonies being black.

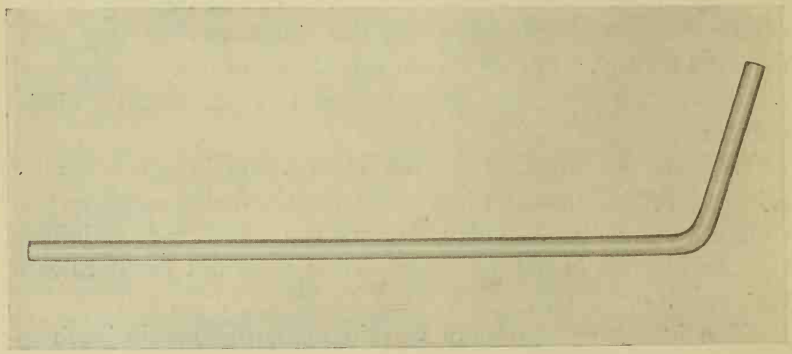

Fig. 2. Bent glass distributing rod.

In brushing these plates of coloured media it is important that the media should be thoroughly dried. A convenient general procedure is to add one loopful of the tube selected for examination to a tube of sterile water. After mixing well place two loopfuls of the latter upon the surface of the L.B.A. or other material selected and distribute uniformly over the plate with a sterile bent glass rod. The medium in the plate will be wet from condensed water on the surface. To dry incubate it for $1\frac{1}{2}$ to 2 hours uncovered in the 37° C. incubator. Then cover and

invert. Incubate for 20—24 hours at 37° C. before the plates are examined.

At least three characteristic colonies should be subcultivated even if they all appear alike. Only one if typical need be worked out, but the others should be subcultivated, so that if the one selected is atypical in any of its characters, these can in turn be fully examined.

The third stage is the application of determining tests. The value of the different tests has already been considered and those advocated for employment are mentioned under the respective substances to be examined.

### *Streptococci.*

In the determination and enumeration of streptococci many of the principles involved are identical with those concerned in the *B. coli* enumeration and explained above so that a shorter consideration is sufficient. As with other indicator organisms a numerical estimation is required, not merely their detection.

There are no known chemical agencies which will enable, by macroscopic appearances alone, tubes of liquid media containing streptococci and other organisms to be differentiated from those which do not contain streptococci. For their preliminary determination in liquid media reliance has therefore to be placed upon other procedures.

When suitable liquid media are employed the presence or absence of streptococci can be judged with considerable accuracy by a careful microscopic examination of the liquid, particularly of any sediment.

Using this basis of enumeration various quantities (0·1, 1·0, 10 c.c., etc.) of the water, milk, or other substance under examination are added to liquid media (such as glucose neutral red broth) which allow the streptococci to grow well and the tubes are carefully microscopically examined after incubation at 37° C. for the presence of streptococci.

It is obvious that such a method is only capable of enabling streptococci as a class, and not the prevalence of any particular kinds, to be enumerated. This method has both the drawbacks of widely spaced results and the errors from unequal distribution

discussed under *B. coli*, while in addition there is possibility of error in that the streptococci may fail to grow or be overgrown or that they may fail to be detected when present.

The last is of some importance and can only be diminished by careful examination in hanging drop preparation, followed in doubtful cases by examination of the stained centrifugalised deposit.

An alternative method, as for *B. coli* enumerations, is to use solid media over which definite fractions of the substance under examination are brushed and then incubated. All colonies likely to be those of streptococci are subcultivated and carefully investigated. Several media have been used as suitable for this purpose, the most useful being ordinary agar and the medium of Drigalski and Conradi.

The enumeration of streptococci by this means is not very satisfactory. Only small quantities (0·1 to 1·0 c.c.) can be used for plating since concentration methods are time consuming and not satisfactory. Also none of the media employed are capable of differentiating in any satisfactory way the probable streptococcus colonies so that in practice a very large number of colonies have to be subcultivated and investigated, very few of which are streptococci. This makes the procedure very tedious. It is also probable that not all streptococci will grow on such plates, particularly when the other colonies are numerous. When it is a question of studying the biological properties of the isolated streptococci this method must be adopted.

The determining tests to apply to isolated streptococci have already been discussed in Chapter I and are further referred to in the subsequent chapters.

### *Bacillus enteritidis sporogenes.*

The estimation is based, not upon the number of these bacilli present, but upon the number of their spores. The cultural feature which is used as pointing to the presumptive presence of this organism is the characteristic change in milk described in Chapter I.

Various amounts of the substances under examination or, if concentration of the bacteria has been effected, definite

equivalent amounts, are added to tubes of sterile whole milk, recently boiled. The destruction of all but spores is ensured by heating to 80° C. and maintaining at that temperature for ten minutes. The milk tubes are then cooled and incubated under anaërobic conditions. The most convenient method of doing this is to employ the pyrogallic acid and caustic potash method. The milk tubes are placed in jars or larger tubes containing pyrogallic acid in powder to which is added, when all is in place, a little 10 per cent. caustic potash solution. The tubes or jars must then be immediately closed by tightly fitting stoppers. The alkaline mixture absorbs the oxygen. The numerical estimation is arrived at by recording the tubes, which show after incubation the characteristic "enteritidis change," as containing the spores of this bacillus while those which do not show it are recorded as negative.

As a rule no attempt is made by workers to isolate the bacillus in pure culture or to definitely prove its presence by animal inoculation. The estimation is therefore not really one of a definite bacillus but that of a group of closely allied bacilli which, as far as is known, have a very similar distribution in nature and a very similar significance.

The estimation as carried out is a fairly simple one and involves no great variations in the hands of individual workers. There are two points in connection with the test which require further consideration. Both deal with the question of the amounts to examine.

It is easy to handle 0·1, 1 and 10 c.c. of the liquid under examination (water, milk, etc.), but when larger amounts have to be examined, as is necessary with water, some method of concentration has to be practised. These concentration of bacteria methods have been mentioned under Streptococci and *B. coli*, but are more conveniently considered here.

To obtain the bacteria in a convenient bulk of fluid it is possible to evaporate off the water at a low temperature under reduced pressure, as in the method for *B. typhosus* in water described in Chapter III, but this is a procedure very rarely employed. The usual plan is to filter the water through a sterile porcelain filter, the water passing through and leaving

the contained bacteria deposited upon the filter. In most cases it is more convenient to filter from within out, so that all the bacteria are deposited upon the inside of the candle. After filtration a definite quantity of sterile water is added and an emulsion made. Usually 10 c.c. represents the final bulk. If, for example, 1000 c.c. of water is concentrated in this way into 10 c.c., it is assumed that each c.c. of the emulsion will contain the bacteria in 100 c.c. of the original fluid and other fractions in proportion. Of course this assumption is not altogether

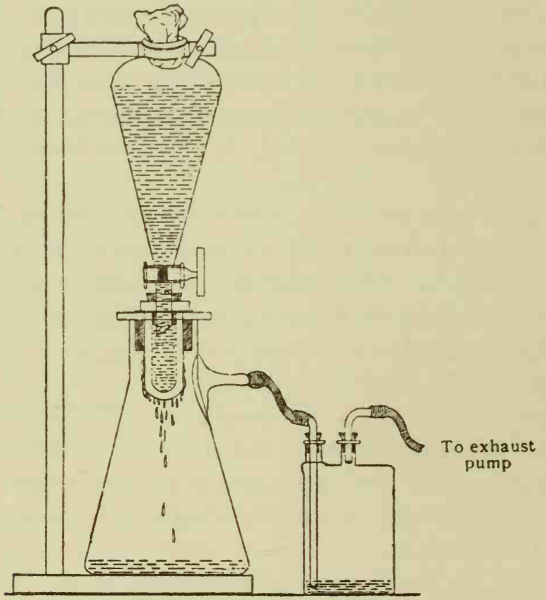


Fig. 3. Apparatus arranged for filtering—aspiration.
From Eyre's *Bacteriological Technique.*

correct, but it is sufficiently accurate for practical purposes. Definite fractions of the emulsion are added to milk tubes and treated as above described.

Filtration is extremely slow through such fine pore filters, so that to accelerate it aspiration or pressure or both may be employed. As a rule aspiration through an exhaust pump is sufficient.

The apparatus figured (Fig. 3) is a convenient form to use. In this apparatus the water is placed in a separating

funnel, which is connected with the filter candle by means of a perforated rubber stopper. The filter flask is connected to an exhaust-pump. In this arrangement the filtration is from within out, so that all the bacteria are deposited upon the inside of the candle. After filtration sterile water is added, and an emulsion made. The porcelain filter tube should hold rather over 10 c.c. of water.

The apparatus must be sterilized in steam for several hours before use.

The actual amounts to select for examination will vary somewhat with the nature of the material being examined. For sewage or milk, for example, only small amounts are required, as these spores are likely to be abundant in such material, while for water 100 or 1000 c.c. are often suitable amounts to examine. As a rule the amounts taken are not nearer spaced than $\frac{1}{10}$, 1, 10, 100, 1000 c.c. etc. This wide spacing, while sufficient for some substances, such as water, is not satisfactory when the spores are likely to be fairly numerous. An alternative and more satisfactory procedure is given under milk (Chapter VI).

*Methods of dilution and of recording results.*

As has already been pointed out, numerical estimations, not mere detection, are essential for the indicator organisms discussed in this chapter. Using the methods recommended, this involves the examination of a series of carefully measured quantities of the substance. Considerable attention to detail is required if reliable and comparable results are to be obtained. When solid substances, for example soil or the meat foods, have to be examined, accurate enumerations of the number of bacteria present or of the number of any special organism are very difficult. The most satisfactory procedure devised up to the present is to add definite weights of the substance to bottles or flasks containing definite amounts of sterile water. As a rule this can most conveniently be done by cautious addition of the soil or other substance to the diluting flask, in equilibrium upon a balance, until the addition of the quantity required is registered. The diluting vessels should have glass

or india-rubber stoppers to enable the solid and the water to be very thoroughly shaken and mixed. Definite fractions of the water are then examined in the same way as for an ordinary fluid and as described below.

The assumption has to be made that the diluting water, after thorough mixture, contains all the bacteria in the solid substance added. This is an inaccurate assumption, since it is not to be anticipated that all the bacteria can be dislodged, the extent of the error varying with the nature of the substance examined. If, however, it is realized that only rough comparable results are in this way obtainable, this method is of great value. The results are most conveniently returned as per gramme of substance.

The procedure is much simpler when liquids have to be directly dealt with, but certain precautions are necessary. The amounts to be examined are removed by sterile pipette. For fractions of a cubic centimetre it is obvious not only that the gradations must be wide apart (*i.e.* the bore must be narrow) but also that too small fractions must not be sampled. When no very special accuracy is required, it is possible to measure sufficiently down to $\frac{1}{10}$ c.c. for water and some other liquids, if they are not heavily contaminated, but with fluids such as milk it is not reliable to take so small a quantity as 0·1 c.c. and errors as great as thirty per cent. may be made in this way. In all cases where possible it is best to obtain these small fractions by making up preliminary dilutions, and this course is absolutely necessary for quantities less than 0·1 c.c.

A whole series of such dilutions may have to be prepared. They are most conveniently made by adding 10 c.c. of the original fluid to 90 c.c. of sterile water in a stoppered bottle, mixing very thoroughly. One c.c. of such a first dilution will represent 0·1 c.c. of the original fluid. In the same way second dilutions can be made from the first, and so on as many as are required. It is preferable to dilute as above rather than to add 1 c.c. to 99 c.c., as obviously there is far less likelihood of errors from slight inaccuracies in measurement.

One mark 10 c.c. pipettes are convenient for making the dilutions in bulk. Such pipettes should be made short (9 inches

or so) for convenience of sterilization, but care should be taken to obtain those in which the lower part is of sufficient length to reach well into the diluting bottles.

While solids are most conveniently recorded as per gramme of material, data as to bacterial content of liquids are returned as per c.c. or litre.

For *B. coli*, streptococci, etc. when a series of dilution tubes are used for enumeration purposes the results are not definite amounts, but can only be returned between the limits of the dilutions employed. For example, if a given water sample showed *B. coli* present in 10 and in 50 c.c. but absent in 1 and 0·1 c.c., the deduction would obviously be that there were more than 100 but less than 1000 *B. coli* per litre of the water and the result would be recorded as *B. coli* 100—1000 per litre. For routine work and reporting this is a preferable method of recording to stating the amounts examined which were positive and those which were negative.

# CHAPTER III

## WATER

Water under natural conditions invariably contains bacteria, the actual numbers present varying enormously with the class of water supply, the degree of soil filtration, opportunities of bacterial contamination, etc.

Natural water supplies as regards their bacterial content are never in a state of equilibrium. On the one hand bacteria-holding substances are constantly being added, thereby raising their bacterial content, while on the other hand certain natural purification agencies are constantly at work tending to reduce the bacteria present. Of these agencies the forces conveniently summed up as sedimentation are the most important, while light, sunlight and deficiency of food material are all factors playing a part. The effects of these different agencies are most readily studied in regard to rivers and streams. An ordinary stream

is constantly fed by small streams containing soil washings, drainage of cultivated and uncultivated land and often drainage from human habitations. In this way vast numbers of bacteria are being added to the water and if samples are collected at favourable situations they show a great increase in the bacterial content. Acting all the while, however, are the agencies mentioned above and making for the diminution of the bacteria present, so that samples taken lower down the same stream may show a much smaller number of bacteria with an absolute, or at least a relative, diminution of the unsatisfactory organisms possibly very prevalent higher up.

Water, unless highly charged with organic matter or unless other conditions are favourable, is not a medium which readily allows bacterial multiplication.

It will be shown later on that the numerical estimation of certain special bacteria is of far greater importance than general bacterial enumerations. The presence of these special bacteria in water supplies is subject to the same influences as affect the total number of bacteria.

In view of the varying influences which affect the bacterial content of water and which are at work unequally for different classes of water it is essential in all cases to carefully consider the nature of the water supply under investigation.

Analysis of water supplies is undertaken for two purposes:

(*a*) To obtain reliable information in regard to the fitness of a given water supply for drinking purposes.

(*b*) To judge the suitability of a supply for domestic or medicinal purposes.

Bacteriology plays no part in the second object, which has to be judged on chemical grounds, but is of very great value in ascertaining suitability for drinking purposes.

From the bacteriological examination of water data can be obtained which not only give information as to existing conditions, but from which deductions can be drawn as to recent harmful pollution.

Bacteriological examinations are also very useful in connection with the testing of the efficient working of sand and other filters.

*Collection and transmission of samples.*

One of the drawbacks of the bacteriological examination of water is the great care which is required in the collection of the samples; even apparently trivial errors or omissions may entirely vitiate the result. Very precise and seemingly trifling directions must be given, unless the sample is collected by an expert.

For the ordinary examination 2-ounce (57 c.c.) glass-stoppered bottles are sufficient.

Fig. 4. Collecting Bottle and Tin.

Sometimes it is advantageous to examine greater quantities of the water and collecting bottles of larger size must be used. It is rarely necessary to examine more than 100 c.c., and this can be most conveniently done by using two of the small bottles for collecting the sample.

If the specimen cannot be examined at once, and delay is unavoidable, the sample should be packed in ice, and then

transmitted to the laboratory. Special forms of apparatus have been designed for this purpose. That figured in Chapter VI under Milk Examination is a very convenient form. As used by the writer, the inner chamber is made to take exactly four bottles and tins as described below.

The bottles fit tins into which they can just slip (Fig. 4). The bottom of the tin is provided with a layer of cotton wool and then a piece of asbestos cardboard. Several thicknesses of asbestos cardboard are also fitted into the cover of the tin so that, when in place, the bottle is firmly in contact with the asbestos above and below. The bottles, with their stoppers rather loosely inserted, should be sterilized in their tins. The tins, after sterilization, are not opened until immediately before the sample is collected.

To take samples from various depths, several different forms of apparatus have been devised. The ordinary collecting bottle may also be used for this purpose. It is tied into a leaden cage, and lowered to the required depth by catgut or string attached to the cage. The loosened stopper is then removed by a jerk upon a second string, previously tied to the stopper, and the sample collected.

In collecting samples from a reservoir, lake or river, plunge below the surface before removing the stopper, thus avoiding scum and surface contaminations. If from wells with pumps, pump away a considerable quantity of water before collecting the sample; while, if a complete investigation is required, a second sample should be obtained after several hours' pumping. If from a tap, allow the water to first run to waste for five to ten minutes. In collecting samples from a tap it is necessary to remember that the interior of the tap may not be clean and care must be taken to obviate this source of error. As far as possible samples should be taken from rising mains and always with as little intervention of pipes as is practicable.

The stopper should only be removed at the actual time of collection, great care being taken that the part of the stopper which goes into the bottle is neither touched nor brought into contact with anything, apart from the bottle or water. It must be held by its free end, and at once replaced and screwed in

firmly when the sample has been collected. The collected water should not quite fill the bottle.

It is essential that full particulars as to the source of the water be supplied with the sample. It is impossible to give a satisfactory opinion without such information.

The following should be recorded:

1. Date of sampling.
2. Nature of the water—spring, upland surface, etc.
3. Whence obtained—pump, draw-well, river, tap, etc.
4. Precise particulars of sampling—*e.g.* depth below surface, from middle or sides. If from tap or pump, time during which the water was allowed to run to waste. Filtered or unfiltered.

If from a tap, note if it was directly connected to a main, or if it was connected with a storage cistern, or other form of supply.

5. Details as to previous rainfall.

The above particulars should all be supplied to the bacteriologist conducting the examination, and in addition to the careful topographical investigation which should be made and recorded in the case of all water supplies.

### *The general nature of the examination.*

Bacteriological water examinations, as conducted at the present day, usually involve three lines of investigation, although all three are not, of necessity, carried out for every sample. These are:

(*a*) The quantitative estimation of the total number of organisms present capable of developing upon the nutrient media used.

(*b*) The isolation and numerical enumeration of organisms not necessarily harmful, but which from their origin are especially liable to be associated with harmful contamination.

(*c*) The isolation and identification of actual disease-producing organisms such as *Bacillus typhosus* and *Spirillum cholerae*.

It will be noticed that from the first two methods deductions only can be drawn as to purity, while with the last method the actual disease-producing organisms are isolated.

### *Quantitative Examination.*

As almost universally practised at the present day, this consists in adding varying quantities of the water to tubes of liquefied gelatine and agar, each of which is then thoroughly mixed and poured out with all precautions into a Petri dish, and solidified as rapidly as possible. These plates (Petri dishes) are then incubated, the agar at 37° C., and the gelatine at 20° C. to 22° C. Each organism present, capable of multiplication under the conditions existing, develops into a mass of bacteria or colony, visible to the naked eye, and as such readily counted. The total number of colonies gives the maximum number of organisms capable of development in the medium used, within the time given and at the particular temperature of incubation.

In actual practice many details have to be attended to. For instance, the reaction of the gelatine and agar used has a marked influence on the number of organisms which develop. Both the English and American Committees on Standard Methods of Water Analysis recommend a + 1 per cent. reaction, and accurately standardized media of this reaction should alone be used for routine work (see Appendix).

The composition of the agar and gelatine nutrient media also influences the number of colonies, and care should be taken to consistently manufacture in the same way and with the same quality and amount of ingredients.

The amount of water to add to the media tubes must obviously vary with the suspected degree of contamination of the water.

For ordinary waters 0·2 and 0·5 c.c. are convenient amounts to add to the gelatine tubes, and 0·2 and 1·0 c.c. to the agar. For contaminated waters a considerably greater dilution may have to be practised. The water is conveniently measured by 1 c.c. pipettes graduated in one-tenths of a c.c., previously

sterilized in the hot-air sterilizer after thorough cleaning and plugging of the upper ends with cotton wool.

In practice proceed as follows: Melt agar and gelatine tubes, and cool down to 42° or 43° C. for the agar, and 25° to 30° C. for the gelatine. Mix the sample thoroughly. Add 0·2 and 0·5 c.c. of the sample respectively to two gelatine tubes, and 0·2 and 1·0 c.c. to agar media tubes. Distribute the water uniformly through the medium by rotation between the fingers. Pour out, after flaming the cotton-wool plugs, into sterilized Petri dishes, only raising the upper dish just sufficiently to admit the top of the test-tube. Solidify as soon as possible either over ice or by using a plate-cooling apparatus (Fig. 5).

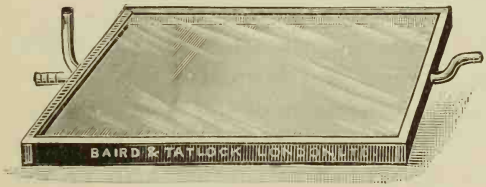


Fig. 5. Savage's Plate-cooling Apparatus.

The inlet pipe is connected by indiarubber tubing to a water tap and the outlet pipe to a sink. Plates of media placed on the thick plate-glass top are rapidly solidified.

Incubate the gelatine plates at 20° to 22° C. and the agar at 37° C. Count the agar plates the next day, and after forty to forty-eight hours. Count the gelatine plates daily, the final count being at the end of seventy-two hours.

To count the colonies it is best to count against a dark background, dividing up the area of the plate, to facilitate counting, by lines on the back made with a paraffin pencil.

All the colonies on the plate should be counted, but if they are very numerous, and an approximate estimation only is possible, then, but only then, some mechanical aid such as Pakes' disc may be used, a few segments counted, and the total number deduced.

In stating results, the details of temperature, time of incubation, and reaction of the medium should always be given and recorded with the analysis.

*Qualitative Examination.*

This may be divided into two parts.

A. The detection and estimation of indicator organisms.

B. The examination for specific disease-producing bacteria.

A. *The detection and estimation of indicator organisms.*

The most important part of the bacteriological examination of water is the examination for and estimation of organisms whose presence serves as an indication of undesirable contamination, such as from excreta or sewage. The value and significance of such indicators has been discussed in Chapter I.

*Examination for B. coli and allied organisms.*

The general principles relating to the isolation of these bacilli together with a consideration of the most suitable methods have been dealt with in Chapter II, so that only the actual procedures recommended for water samples need to be described. The following is recommended as convenient and satisfactory:

Add 0·1 and 1·0 c.c. respectively to tubes of lactose bile salt broth in double tubes. Add 10 c.c. to a similar tube, but containing lactose bile salt broth of double strength. To the remainder in the bottle, after all the different amounts of water have been withdrawn for the different parts of the examination, add the contents (about 10 c.c.) of a tube of four times strength neutral red broth. Replace the glass stopper. Four times strength bile salt broth may be used, and, if the examination is for *B. coli* alone, is preferable, but by using neutral red broth the mixture is also available for the examination for streptococci.

If a 2-ounce sample is collected, the amount remaining in the bottle will be about 30 c.c. If a large sample of water is collected, then 50 c.c. should be added by sterile pipette to a tube of four times strength neutral red broth large enough to hold the added water.

The tubes are labelled, incubated at 37° C., and examined after twenty-four and after forty-eight hours.

If the 0·1, 1·0, and 10 c.c. tubes show no gas after forty-eight hours, it can be assumed that *B. coli* is absent in these amounts. Then, in every case, the larger amount (*i.e.* the 30 c.c. in the bottle) should be examined for this organism. The alteration of the red colour to yellow, with the presence of fluorescence, is an indication of the probable presence of *B. coli.*

If gas is present in the tubes containing smaller amounts, use the one showing gas in the tube with the least quantity of added water for inoculating plates of solid media. In this way it can be definitely ascertained whether *B. coli* is present or absent in 50 c.c. or less, and if present, approximately in what numbers.

In certain cases it is of value to ascertain the presence or absence of *B. coli* in 500 or 1000 c.c. of the water.

This may be done by filtering the water through a porcelain filter, as described for the examination of *B. enteritidis sporogenes* (Chapter II), and using the filter brushings, emulsified in a little water, to add to tubes of bile salt broth. This method is, however, cumbersome and unsatisfactory.

A far better procedure is to convert the water sample itself into a nutrient medium by the addition of four times strength broth, incubate at 37° C. for twenty-four hours, and then either inoculate suitable plates direct, or, what is preferable, add 1 c.c. by sterile pipette to a tube of bile salt broth, and incubate this for one or two days. If any *B. coli* were present in the original bulk of water, the preliminary incubation in the water broth would have allowed them to multiply sufficiently to be at least present in 1 c.c. of the sample, an amount of fluid readily examined.

The composition of the media used is given in the Appendix.

To isolate the *B. coli* group organism a trace of the positive tube selected is distributed over the surface of a plate containing neutral red lactose bile salt agar (L.B.A.), fuchsin agar or other medium selected. L.B.A. is recommended as most suitable. Several colonies should be subcultivated and worked out.

Subcultivation upon or in the following five media is recommended for routine work, *i.e.*:

(*a*) Gelatine slope (for morphology, motility, cultural appearance, and liquefaction).

(*b*) Litmus-milk at 37° C.

(*c*) Lactose-peptone litmus solution (in a double tube).

(*d*) Peptone water (for indol production).

(*e*) Saccharose peptone litmus solution (in a double tube).

Houston[1] has suggested and used very extensively a number of modifications which are valuable when a large series of samples have to be examined. The *B. coli* organisms are isolated from the positive gas tubes by inoculating sloped lactose, bile salt, neutral red peptone agar (called by Houston rebipelagar) in test tubes instead of plates. As far as possible five red colonies are in each case subcultivated and investigated. The confirmatory tests selected are carried out with the following media :—glucose litmus gelatine, lactose litmus gelatine, saccharose litmus gelatine and peptone water. The results are recorded after 24 hours' incubation at 20—22° C., the data recorded being the production of gas from the three sugars and the presence of indol.

Houston recommends gelatine sugar media (1 p.c. sugar, 2 p.c. peptone, 1 p.c. lemco, 7·5 p.c. gelatine and 1 c.c. of a 5 p.c. potassium hydrate solution) for gas production testing, finding such media more sensitive than the usual liquid media.

Houston has introduced a number of modifications which save time when dealing with large batches of routine samples such as are examined daily in the Metropolitan Water Board Laboratories.

Labelling is reduced to a minimum by the use of coloured cotton wool plugs. Five colonies from each rebipelagar tube being subcultivated the media are tested in sets of five. Five quite small test tubes ($2'' \times \frac{1}{4}''$) filled with the same sugar gelatine or other medium used are placed, in one large test tube, only the latter having a cotton-wool plug.

To expedite the inoculations the colony selected is picked off by a sterile straight iron wire (sterilized in batches over naked bunsen flame). The wire is then placed in a tiny test tube containing a few drops of sterile salt solution. Into this little tube are then introduced as many more sterile wires as

[1] For a more detailed account see *Metropolitan Water Supply Report* for January, 1907, pp. 46–52.

correspond to the number of tests proposed to be made and these are used to inoculate the confirmatory tests. This saves time and trouble, besides avoiding any uncertainty in repeatedly going back to a colony in order to inoculate a series of cultures.

### *Examination for streptococci.*

The detection of the presence of streptococci and their numerical estimation in water is a procedure which is only carried out by some bacteriologists but is one which is decidedly useful.

The method originally employed by Houston, to whom is due the credit of first pointing out their importance in water examinations, was to concentrate the water by passing a litre or other definite quantity through a sterile porcelain filter and brush definite fractions over agar plates. The agar plates were examined after 24 hours' incubation at 37° C. and the minute colonies subcultivated into broth tubes and further investigated, if streptococci were found to have grown. In more recent work, examining London water, Houston brushed 1 c.c. of the water directly over plates containing Drigalski-Conradi medium and subcultivated all the minute colonies which developed after incubation at 37° C.

The differentiating tests to apply are discussed in Chapter I.

The method employed by the writer is based upon the detection of streptococcus chains in different fractions of the water sample.

This may conveniently be done by adding 0·1 and 1·0 c.c. respectively to tubes of glucose neutral red broth, and 10 c.c. to a tube of the same broth, but of double strength. These, together with the 30 c.c. preparation used in the *B. coli* examination, are examined after incubation at 37° C. for 40 to 48 hours in hanging-drop preparation. Only cocci in quite definite chains can be taken as evidence of the presence of streptococci, and several preparations should be examined from each tube.

It is true that in this way the presence of streptococci *as a class* only will be ascertained, but with our present knowledge the streptococcus test for excretal contamination does not rest upon

the presence of any one variety of streptococcus, but upon the group as a whole.

Neutral red broth is preferred to plain nutrient broth because the streptococcus chains seem to be more readily detected microscopically in it.

In cases in which it is doubtful whether streptococci in chains are actually present a definite opinion may often be arrived at by centrifugalizing the fluid, and microscopically examining a little of the deposit stained by methylene blue.

*Examination for B. enteritidis sporogenes.*

This examination is carried out by some bacteriologists and is included here although not recommended.

Even in waters from contaminated sources this organism may be present in only small numbers, so that it is usually necessary to examine a considerable volume of the water. Convenient amounts to examine are 10, 100, 500 and 1000 c.c.

To deal with these large volumes of water the sample is concentrated by filtration through porcelain as described in Chapter II.

If one litre is concentrated into 10 c.c., then 10, 100 and 500 c.c. of the original water will be represented by 0·1, 1 and 5 c.c. respectively of the concentrated filtrate.

The presence of the spores of this bacillus are determined by inoculating milk tubes as described in Chapter II.

B. *The examination for specific disease-producing bacilli.*

Some diseases, such as diphtheria or scarlet fever, appear to be never spread through water. The only bacilli associated with definite diseases for which it is ever necessary to examine water are the typhoid bacillus, the cholera vibrio, the organisms of dysentery in the tropics, and, under very exceptional circumstances, members of the Gaertner group in suspected outbreaks of food poisoning or paratyphoid fever. The examination for Gaertner group organisms is described in Chapter VIII and modifications for the examination of water will readily suggest themselves. Only the examination for the bacteria of

typhoid fever and cholera will therefore be dealt with in this chapter.

### *Examination for B. typhosus.*

The methods available for the examination of water for the typhoid bacillus are still not very satisfactory, and this is evidenced by the very large number which have been recommended. Great improvements in procedure have taken place in recent years resulting from the introduction of selective differentiating media and of more satisfactory methods of concentrating the bacilli.

The problem is a difficult one, because a comparatively delicate organism, with no very definite morphological or cultural characters, has to be detected among a large number of other organisms which for the most part thrive much better on all media and at all temperatures than itself.

Further, the organism may be present in very small numbers, for it tends to rapidly die out in water, so that it is necessary to examine a large bulk of water.

Moreover, owing to the long incubation period of the disease, attention is usually not directed to the water as a possible source of the infection until several weeks after the specific contamination, and when all the typhoid bacilli may have died out.

The identification of *B. typhosus* from water naturally divides itself into three stages:

1. Preliminary methods, whereby any typhoid bacilli present are obtained in a quantity of fluid small enough to be directly plated.

2. The isolation of the organism in pure culture.

3. The tests necessary to establish its identity.

1. *Concentration.* One or other of the following preliminary methods may be used:

(*a*) *Enrichment.* By the addition of concentrated broth or other substance to the water, it may be converted into a nutrient medium in which any contained typhoid bacilli can multiply so as to be, after incubation, numerous enough to be present in a very small quantity of the fluid. If only plain nutrient broth is

used, there is considerable danger that some of the other bacilli present will multiply at a greater rate than any typhoid bacilli present, and the latter be relatively less numerous after incubation, if not actually suppressed. Numerous chemical substances have been tried, or advocated, as enabling the typhoid bacillus to multiply relatively better than competing bacilli, but most of them, on further investigation, have not been found to be reliable. Malachite green has been found to be of some service, but a series of different dilutions must be employed. The water is placed in flasks, and sufficient malachite green broth is added to each to make the strength of malachite green in the mixture 1 in 2000, 1 in 5000, 1 in 10,000 respectively. After incubation at 37° C., or preferably at 40° to 42° C., for 24 hours, the mixtures are plated upon the solid media described below.

On the whole it may be said that enrichment and selective enrichment methods are less satisfactory than sedimentation or direct concentration.

(*b*) *Concentration by mechanical precipitation by chemicals.* Chemicals are added which are harmless to typhoid bacilli but which form a flocculent precipitate which carries down all or most of the bacilli in the water and enables them to be obtained in a small and easily handled bulk of material. If a centrifuge is available precipitation is facilitated.

A number of chemicals have been recommended for this purpose, *e.g.* lead acetate and sodium hyposulphite (Vallet and Schüder), iron sulphate (Ficker), alum (Willson), liquor ferri oxychlorati (Müller).

Ficker's method is perhaps as good as any. In this method, two litres of the water are placed in a tall cylinder, and mixed with 8 c.c. of a 10 per cent. soda solution. Seven c.c. of a 10 per cent. solution of ferrous sulphate are then added, and stirred in with a glass rod. The mixture is placed in an ice-chest, and allowed to stand for a few hours; if a centrifuge is available the precipitate can be separated at once. The precipitate is transferred to a sterile tube, and about half its volume of a 25 per cent. solution of neutral potassium tartrate is added. The tube is corked and well shaken, until the precipitate is completely dissolved, more tartrate being added if necessary. From this

solution large Petri dishes containing Drigalski-Conradi or other suitable solid media are inoculated.

Ficker claims that by this method there is very little loss of typhoid bacilli; 97 to 98 per cent. of those present being carried down with the precipitate.

(*c*) *Concentration by sedimentation without chemical precipitation.* If a powerful centrifuge is available the water may be directly centrifugalized and the deposit used for plating. Houston[1] in some investigations upon typhoid bacilli in river water used this method. In dealing with a raw water containing 72,000 bacteria per c.c. he was able to recover the typhoid bacillus when 47 had been added to 100 c.c. of the water.

(*d*) *Concentration by evaporation under reduced pressure.* Wilson[2] has described a method which enables water to be

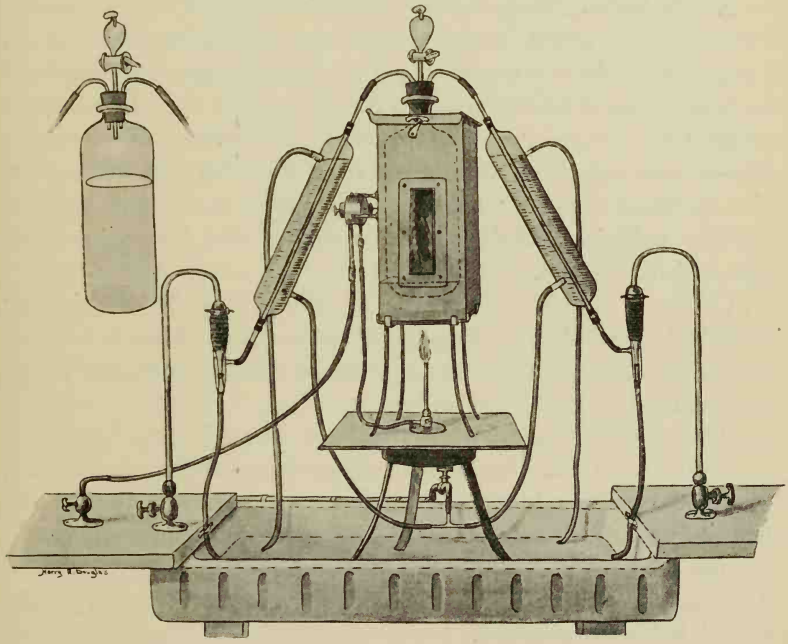

Fig. 6. Wilson's apparatus for concentration under reduced pressure.

[1] *Fifth Research Report, Metropolitan Water Board,* 1910.

[2] *Brit. Med. Journ.* May 18, 1907.

rapidly concentrated without destruction of bacteria. The glass reservoir containing the sample of water is connected with an exhaust pump which rapidly lowers the pressure. The reservoir is immersed in water which is accurately kept at 37° C. The water boils at this reduced temperature and as a rule 24 hours suffice to evaporate four litres of water almost to dryness. The residue is spread over solid media plates as described below.

This method can also be combined with "enrichment" by the addition of broth or other nutrient medium to the water two hours after the commencement of the evaporation, this delay being necessary to avoid frothing.

In a later paper Wilson and Dickson record that they were able, using this method, to recover the typhoid bacillus from water containing only 30 typhoid bacilli in four litres, equivalent to one bacillus in 133 c.c. of water.

2. *Isolation.* To isolate the typhoid bacilli from the concentrated material a large number of special media are now available. These media do not so much show which colonies are typhoid bacillus colonies as indicate the colonies which certainly are not typhoid bacillus colonies. In this way a vast number of organisms also present are either suppressed or, if they develop, are readily distinguished by their colour, appearance, etc., from typhoid bacillus colonies. *All* colonies possibly the latter must be further investigated.

Of these special media, the following may be mentioned: Drigalski-Conradi agar, lactose bile salt neutral red agar (L.B.A.), fuchsin agar, malachite green agar and brilliant green picric acid agar as modified by Fawcus.

The composition of these media is given in the Appendix.

Houston in the investigations mentioned above used L.B.A. modified by the addition of other sugars, etc. In addition to lactose he added saccharose, adonite, raffinose and salicin, all 0·2 per cent. of each. By their addition the number of white colonies was still further reduced and without risk of typhoid bacilli being overlooked.

With all these media the plates before or after inoculation should be thoroughly dried uncovered, preferably in the incubator. They are then covered, inverted and incubated. The

colonies are sufficiently differentiated after 16 to 24 hours at 37° C.

With Drigalski-Conradi agar the *B. coli* colonies are red, not transparent, and have a diameter of 2 to 6 millimetres, but considerable variation in size and degree of colour are met with. The *B. typhosus* colonies are blue, with a violet tinge; they are transparent and resemble dewdrops, and have a diameter of 1 to 3 millimetres, seldom larger.

With fuchsin agar the *B. coli* colonies are bright red, round and have prominent margins; the typhoid colonies are round, colourless, very transparent, and have thin margins.

With L.B.A. *B. coli* and other lactose fermenters grow as red colonies while *B. typhosus*, Gaertner group bacilli and other non-lactose fermenters form white colonies.

Using Fawcus's brilliant green picric acid agar lactose fermenters form opaque colonies, while *B. typhosus* and other non-lactose fermenters grow as clear colonies.

Malachite green agar is more suitable for Gaertner group work. Fuchsin agar does not keep well and Drigalski-Conradi agar is troublesome to prepare and is not always satisfactory to use. Fawcus's medium is praised by some workers but has not been very satisfactory in the writer's hands.

In general L.B.A., with or without the addition of further sugars, etc., is recommended as the most generally useful, while fuchsin agar is also decidedly valuable.

3. *Identification.* *All* suspicious colonies found on the different media in the plates are subcultivated into broth, and incubated at 37° C. until next day. They are all then examined in hanging drop, and those which show actively motile bacilli are tested with anti-typhoid serum. A fairly powerful serum should be available, and a dilution of not less than one per cent. should be employed.

A quicker and sometimes preferable method is to directly test with the antityphoid serum each of the selected colonies by rubbing up a little of the colony in a drop of one per cent. serum on a coverglass. Only those which contain reacting bacilli are subcultivated.

All those which fail to show agglutination are rejected, while

the tubes or colonies reacting are each subcultivated into litmus-milk, glucose litmus broth (in a double tube), and lactose-peptone solution (in a double tube).

All the organisms giving cultural characters in these media which accord with those of *B. typhosus* are then fully worked out. The tests should include accurate and extended agglutination tests with highly dilute sera.

Some such procedure as the above will rapidly decide whether any typhoid bacilli have been isolated.

### *Examination for Spirillum cholerae.*

Of the different methods suggested, the simplest and most satisfactory is the enrichment method employed by Koch and others. This is best done by converting the water itself into a nutrient medium by the addition of peptone and salt.

About a litre of the water is placed in twelve large sterile flasks, 90 c.c. in each. To each is added 10 c.c. of a sterile solution, consisting of 10 per cent. peptone, and 5 per cent. sodium chloride. The flasks are then incubated at 37° C. An alternative plan is to use only one flask containing 1 litre of the water and to add the peptone salt solution to this. Even quantities of ten litres can be treated in this way or, if incubators of sufficient size are not available, the water can be dealt with in a series of smaller bottles (2 to 4 litre capacity). After six, twelve and eighteen hours' incubation, microscopic preparations and examinations in hanging drop are made from the surface of each flask. The medium is one in which the cholera spirillum grows very rapidly, and if present, is found in the very thin pellicle on the surface of the liquid.

According to Koch, after six hours' incubation is the most favourable time to examine but sometimes it is necessary to wait longer. The flasks which show the presence of vibrios are used to inoculate plates of the media selected, a loopful of the fluid being withdrawn from the surface for this purpose.

When large quantities of water are examined some of the concentration methods described as suitable for the isolation of the typhoid bacillus may be used.

Agar and gelatine media have been most used for the isolation, but recently a large number of special media have been advocated for this purpose. While a number of them are useful, that of Dieudonné, published in 1909, would appear to be most valuable. It is an alkaline blood agar medium the exact preparation of which is described in the Appendix.

The plates ought not to be used immediately after their preparation. Dieudonné recommends keeping them for several days in the incubator at 37° C. uncovered and face down, or to heat them for five minutes at 65° C. According to the Report of the International Commission[1], an equally good result is obtained by keeping them for 48 hours at laboratory temperature. During this time the surface of the agar becomes slightly dry and loses a part of its alkalinity. Once in condition the plates ought to be used within a period not exceeding five or six days. Cholera vibrios grow abundantly on this medium while the bacilli of typhoid fever and dysentery and in particular the *B. coli* group organisms grow either very badly or not at all.

On the other hand, *B. proteus* and *B. pyocyaneus*, both encountered frequently in diarrhroeal stools, grow nearly as well as the cholera vibrio and considerably complicate the search. Also many of the non-choleraic vibrios found in water and excreta appear to grow in this medium. According to Dieudonné, however, most of these vibrios are suppressed.

The colonies of the cholera vibrio on this medium are transparent and greyish with a glistening appearance by reflected light but are not definitely characteristic. When many other bacteria are present the identification of the cholera colonies may be very difficult.

Ordinary nutrient agar is superior to gelatine but the agar must be alkaline. The agar colonies are not very distinctive, being flat discs, transparent and of a grey-blue colour.

All suspicious colonies on the agar, Dieudonné agar or other medium used, are subcultivated and their characters and properties studied in pure culture. An alternative plan and one which

[1] "Report presented to the permanent committee of the International Office of Public Hygiene in the name of a Commission presided over by Dr Ruffer." Translation: *U. S. A. Public Health Reports*, 1912, vol. XXVII, p. 371.

saves time, but not labour, is to directly test every suspicious and possible colony with powerful anti-cholera serum. All the vibrios which are agglutinated are subcultivated and their cultural characters and serological properties tested in detail.

Houston[1] in his investigations upon the vitality of cholera vibrios in water and their isolation therefrom used agar, bile-salt agar and Drigalski-Conradi agar. The bile salt however considerably inhibits growth and only very small red colonies are produced by the cholera vibrio.

If a large number of possible colonies have to be sorted out, it is essential to have a few cultural tests which rapidly eliminate the majority of those which are not cholera vibrios. Houston's procedure was to subcultivate all possible colonies into saccharose peptone water in double tubes. Only those which showed acid without gas formation after 24 hours at 37° C. were further investigated. Other important eliminating tests used by him were growth in peptone water (for cholera-red reaction) and upon gelatine slope. Failure to give a cholera-red reaction in 24 and in 48 hours, and *rapid* liquefaction of gelatine were sufficient to exclude the organisms as not cholera vibrios. The organisms which passed these tests were subjected to further cultural investigation.

Since a considerable number of vibrios which closely resemble the true cholera organism have been found in water it is essential that all available tests be employed before an organism is accepted as *Sp. cholerae.*

Morphological, cultural and pathogenicity tests are valuable in the diagnosis of cholera vibrios but they are not sufficient in themselves, and vibrios have repeatedly been isolated from water which, as regards these tests, cannot be distinguished from those isolated from the intestines of cholera cases but which on other grounds cannot be considered true cholera vibrios.

By the use of additional tests, particularly agglutination tests and the immunity reactions of Pfeiffer and of Bordet, accurate differentiation can be arrived at.

The International Office Report above referred to lays down the following in regard to agglutination tests, based upon the

[1] *Fourth and Fifth Research Reports, Metropolitan Water Board.*

work of Kolle and Gotschlich and others. This Report states, "It appears, therefore, that a rule might with advantage be adopted to regard as choleraic every vibrio which is agglutinated in 1—1000 at least by a serum the activity of which is 1—4000 or over. For vibrios agglutinable by a cholera serum only in stronger dilutions (1 : 500 to 1 : 1000) the results should be considered as doubtful."

Other tests which have been shown to be of value are the haemolysis test and estimation of deviation of complement.

A description of these tests would be out of place here, and general and special text-books of bacteriology must be consulted.

*Significance and interpretation of results.*

The detection of the cholera spirillum or the typhoid bacillus in a water, in whatever amount, is sufficient to condemn the supply. The other results obtained in the bacteriological examination of water supplies are, however, only data from which an opinion upon the purity or contamination of the water can be deduced with more or less confidence according to the facts available.

Such deductions require much special experience and a careful consideration of a number of facts, some of which will be briefly discussed.

As already explained, the contamination of water with excreta or sewage is mainly dangerous (although not entirely so) from the possibility that sooner or later it may be associated with contamination with excrementitious matters containing the typhoid bacillus and in this way an outbreak of enteric fever be set up. Such specific pollution with the typhoid bacillus is almost invariably associated with the addition of *B. coli* and streptococci to the water, and while the typhoid bacillus is not readily detected the existence of danger may be shown by these indicator organisms. The vitality of *B. typhosus* in water and particularly the relative vitality of this bacillus and *B. coli* in water is therefore of great practical importance. On its negative side it is necessary to know how far the absence or relative absence of bacilli of the *B. coli* group can be accepted as presumptive proof that typhoid

bacilli are absent and the supply in its present condition a safe one.

*Viability of B. typhosus in water.*

The viability of the typhoid bacillus in water has been made the subject of many investigations. Only a few of the more recent and extended can be mentioned here.

The results of many of the investigations are conflicting. In explanation of the discrepancies several points may be urged. In the first place, many of the experiments have been carried out under highly artificial conditions. For example, the bacilli have been added to water small in bulk and kept confined in vessels, such as in flasks or bottles. Typhoid bacilli in such circumstances are not under conditions of light, movement and competition with naturally occurring bacilli such as must take place under natural conditions. Deductions from such experiments furnish no real guide as to the viability of the typhoid bacillus under actual practical conditions of pollution of a water supply by material containing this bacillus. When water supplies are contaminated with typhoid bacilli the vehicle of infection is usually the urine or faeces of cases. Such material generally contains vast numbers of other bacilli. It is also probable that the viability of any added bacilli is considerably influenced by the addition of the considerable amount of organic matter which usually in this way gains access to the water.

A further factor of undoubted importance is the individual resistance of the bacilli added to the water. Many of the earlier experiments were conducted with typhoid bacilli which had been cultivated in the laboratory outside the animal body for long periods. Houston[1] found that typhoid bacilli direct from the animal organism died much more rapidly in river water than strains previously cultivated in the laboratory. In 13 experiments with the "uncultivated" typhoid bacilli the bacilli could not be found in the infected water after one week (9 experiments), after two weeks (3 experiments) and after three weeks (1 experiment): yet the same microbe after "cultivation" usually lived over five weeks.

[1] *Seventh Research Report*, p. 3.

Of recent investigations upon this subject the following are two of the most important.

Jordan, Russell and Zeit[1] carried out three independent series of experiments which later were re-investigated by Russell and Fuller[2]. They used recently isolated *B. typhosus* strains and the bacilli were suspended in the water in celloidin or parchment sacs. The waters used for experiment were lake and river water and the impure Chicago Drainage Canal water.

Jordan, Russell and Zeit concluded that "under conditions that probably closely simulate those in nature, the vast majority of typhoid bacilli introduced into the several waters studied, perished within three to four days." They however suggested that specially resistant cells may be able to live for longer periods.

Their experiments show the relative rather than the ultimate disappearance of the bacilli, since the whole bulk of fluid was not examined for the typhoid bacillus but only one c.c. or other small amount.

Houston[3] has recently carried out some extended investigations. In one series "cultivated" bacilli were added to *raw* river water stored in the laboratory in partially filled stoppered bottles. In these experiments the vast majority of the bacilli perished within one week, but a few specially resistant strains persisted for several weeks, the final extinction of the bacilli (as judged by inability to isolate it from 100 c.c. of the water) only taking place after nine weeks.

In a second series the typhoid bacilli were added to the water direct from actual typhoid bacilli carrier cases, the bacilli being added either directly centrifugalized from urine or in the urine itself.

In the first experiment the first week effected a percentage reduction of 99·99 to 100 in the number of typhoid bacilli, their ultimate death (as judged by inability to isolate it from 100 c.c.) taking place either within one week or by the second week. Subsequent experiments gave similar results but in one experiment, using an outdoor tank, the uncultivated bacilli were still found in 100 c.c. after three weeks.

[1] *Journ. of Infectious Diseases*, 1904, vol. 1, p. 641.

[2] *Ibid.* Supplement No. 2, Feb. 1906, p. 40.

[3] *Seventh Research Report.*

Houston also carried out some experiments upon the comparative vitality of the typhoid bacillus in raw Thames river water at different temperatures. He found that temperature exerted a powerful influence, the bacillus living considerably longer at low (32—42° F.) than at higher (50—98·6° F.) temperatures.

It may be said, as a general result from the experimental evidence, that typhoid bacilli do not live for long periods in drinking water, their total elimination being a matter of only a few weeks, while the vast majority are destroyed within a few days. At the same time it is hardly justifiable to assume that all typhoid bacilli gaining access to water will be eliminated even within a month in all classes of water. Such a conclusion is probably true as applied to the storage of raw river water, but with local well water contaminated with the faeces of a typhoid fever case or carrier the conditions—particularly as regards the nutritive condition of the water—are considerably altered from those made use of in the experimental work and deductions from these experiments may not apply. It is quite possible that the bacilli may persist for considerably longer periods.

Wilson and Dickson[1] added a mixture of typhoid "carrier" urine and faeces to 60 litres of water in an open reservoir and recovered the typhoid bacillus after three weeks and two days.

As regards the comparative vitality of *B. typhosus* and *B. coli* in water, comparative results show clearly that the former is the more delicate organism and dies out first. The proved absence of the second organism may therefore be taken—since almost invariably contamination with *B. typhosus* is accompanied by much heavier contamination with *B. coli*—as evidence of the absence of the typhoid bacillus. The only exception would be when the contamination was by the urine of a typhoid case or carrier containing only typhoid bacilli and no *B. coli*.

### *Bacterial content of different classes of waters.*

Before the significance of the results of the bacteriological examination of water samples can be properly appreciated

[1] *Journ. Royal Sanitary Instit.* 1911, vol. XXXII, p. 472.

certain general and special facts in regard to the bacterial content of different classes of waters must be considered.

For present purposes water supplies may be divided into the following groups:

A. Upland surface waters.

B. Deep water supplies—deep wells and springs.

C. Shallow wells and subsoil waters.

D. River water.

Each class of water must be separately considered.

### *Upland surface waters.*

Supplies of this class are derived from rain water which has been in contact with soil, usually uncultivated soil, but which have not undergone any filtration through soil. Rain water itself when pure contains but few bacteria and no excretal indicator organisms. The bacterial content of upland waters will vary with the bacterial nature of the soil they wash, the degree to which they are liable to pollution from sources other than soil and to some extent with the degree of storage to which they have been subjected.

Upland uncultivated soil may contain numerous bacilli but is free from *B. coli* and other indicator organisms. The bacterial content of water in contact with such soil may be considerable but it will not contain *B. coli.*

Moorland soil of this character is however usually used for grazing to some extent and is the habitat of wild fowl and other birds. The excreta of these birds and animals contain numerous *B. coli* and other indicator organisms and thus serve as a source of such organisms to the water. As a matter of practical experience therefore it is found that many upland surface waters contain these indicator bacilli in considerable and sometimes in large numbers, and this even when the uplands which serve as the gathering area are remote from human habitations and free from all risk of contamination from human sources.

As illustrations of results obtained with such waters in which

topographical examination showed no possibility of human contamination the following may be quoted :

| Source[1] | | organisms per c.c. | | *B. coli* group organisms in | | | |
|---|---|---|---|---|---|---|---|
| | | 37° C. | 20° C. | 0·5 | 2 | 10 | 40 c.c. |
| Pure upland surface supply on old red sandstone. Sheep and other animals grazed but no other source of contamination | Main contributory river. | 4 | 226 | − | − | + | + |
| | ,, ,, ,, | 3 | 218 | − | − | − | + |
| | ,, ,, ,, ¾ mile above the previous two samples ... ... | 2 | 188 | − | − | + | + |
| | Spring rising near ... | 4 | 458 | − | − | − | − |
| Large upland supply with two main collecting reservoirs. Liable to sheep and bird contamination but not to drainage from cultivated land or other sources | 345 mil. gallon reservoir | 8 | 218 | − | − | + | + |
| | | 42 | 850 | − | + | + | + |
| | 222 ,, ,, ,, | 36 | 410 | − | + | + | + |
| | | 65 | 995 | − | + | + | + |
| | Main stream entering reservoir ... ... | 132 | 350 | − | − | − | + |
| | Small stream entering reservoir ... ... | 4 | 200 | − | − | − | + |

It is obvious that for this class of waters bacteriological analyses must be interpreted with considerable caution and only in the light of all the topographical and other data available. While the addition of animal excreta is not of anything like the same potential harmfulness as human excreta, yet in considerable amount it is unsatisfactory and should be prevented. It is therefore justifiable to look adversely upon water supplies of this character which contain a large number of *B. coli* and possibly other indicator organisms. Each case requires careful consideration but in general the presence of 'excretal' *B. coli* in 1 c.c. or less of such waters points to heavy and undesirable pollution while if less numerous special topographical investigation is required.

### *Deep water supplies.*

Waters of this class—deep well or spring—are derived from rain which has filtered through a considerable depth of soil and which has usually taken a considerable time over the process. The deeper layers of soil are germ free and in its passage the bacilli are filtered out of the water.

[1] For a detailed consideration of the bacterial content of upland surface waters, especially in relation to topographical findings, see Savage, 1902, *Journ. of Hygiene*, II, p. 320.

Deep well waters when uncontaminated contain but few bacilli and no *B. coli* or other indicator organisms. In waters of this class therefore a considerable increase in the number of organisms and in particular the presence of *B. coli*, with or without streptococci, must indicate pollution with surface water or other undesirable material and much higher standards of requirements are reasonable. It is justifiable to maintain an attitude of great suspicion towards any water from such sources which contains *B. coli* in 100 c.c. or less.

To ascertain the precise source of contamination and to measure and assess its danger often requires a very careful study of topographical and geological conditions and the making of a series of subsidiary bacteriological examinations.

One important cause of bad bacteriological results from deep water supplies, which it is important to eliminate or confirm at the outset, is contamination of the supply at the surface outcrop.

Frequently a spring runs some distance on the surface before it is collected and utilised, and samples taken at a point on the distribution side may entirely owe their bad bacteriological results to surface contamination of an otherwise pure deep water supply.

It is important to realize how frequently such surface contamination occurs and is allowed to exist owing to the carelessness or want of knowledge on the part of those entrusted with the care of the water supply. The following is a striking illustration of such contamination and its influence upon the bacteriological examination results.

Samples were received in November 1912 from three separate springs in connection with a proposed new water supply. All three springs were from the same formation and indeed from the same hill. No. 2 was satisfactory while No. 1 and No. 3 showed evidence of contamination. A careful examination of the local conditions was recommended. At first it seemed difficult to account for any difference in quality since the three springs were obviously coming from the same strata and in each case were piped for about 100 yards from the springs, glazed open-jointed earthenware pipes being used. Further examination however disclosed the fact that in springs 1 and 3 the pipes had been covered with a layer of bracken by the workmen employed

(contrary to instructions) and the trenches were not filled with rock and earth as carried out for spring No. 2. When this bracken was removed and the trench satisfactorily filled with rock and earth these springs yielded bacteriologically pure water.

The analyses made were as follows:

| Source | Date | Organisms per c.c. at 37° C. | Organisms per c.c. at 21° C. | *B. coli* (per litre) | *Streptococci* (per litre) |
|---|---|---|---|---|---|
| No. 1 Spring | Nov. 1912 | 6 | 30 | 1000—10,000 (atypical) | 1000—10,000 |
| ,, 2 ,, | ,, ,, | 13 | 35 | absent in 50 c.c. | absent in 50 c.c. |
| ,, 3 ,, | ,, ,, | 8 | 25 | 1000—10,000 (atypical) | 100—1000 |
| ,, 1 ,, | March 1913 | 2 | 5 | absent in 50 c.c. | absent in 50 c.c. |
| ,, 3 ,, | ,, ,, | 1 | 4 | ,, ,, | ,, ,, |

Admixture with surface water is particularly liable to occur in samples of spring water taken in connection with proposed new sources of water supply, since in such cases usually the only samples which can be collected without expense are those admixed with surface water where the spring outcrops.

The following is an interesting illustration of the value of careful investigation of deep water supplies and the use of bacteriology to detect surface contamination.

The public water supply of a small town showed on bacteriological examination distinct evidence of contamination (sample No. 1 below). The supply was derived from three springs in the old red sandstone, the water being conveyed to a brick-covered reservoir (36,000 gallons capacity) by *agricultural* pipes. A separate analysis was made of the water from each spring (Nos. 2, 3 and 4) while a further analysis of the water in the reservoir (No. 5) was still not satisfactory. The springs were, as shown from these and other analyses, mostly satisfactory and the unsatisfactory condition of the supply was due to surface water passing into the pipes and so into the main supply. Another spring known to be liable to some contamination and excluded from the supply was examined (No. 6) at the same time and shown to be unsatisfactory. Steps were taken to put in proper pipes and cut off all surface water and the latest analysis (No. 7) was quite satisfactory.

| No. | Source | Date of Exam. | Organisms growing at 37° | Organisms growing at 21° | *B. coli* (per litre) | *Streptococci* (per litre) |
|---|---|---|---|---|---|---|
| 1 | Service reservoir | Jan. 1912 | 110 | 250 | 1000—10,000 | 30—100 |
| 2 | Spring *A* | Feb. ,, | 4 | 30 | absent in 50 c.c. | absent in 50 c.c. |
| 3 | ,, *B* | ,, ,, | 3 | 21 | 30—100 | ,, ,, |
| 4 | ,, *C* | ,, ,, | 9 | 80 | absent in 50 c.c. | ,, ,, |
| 5 | Service reservoir | ,, ,, | 40 | 120 | 30—100 | ,, ,, |
| 6 | Spring *D* | ,, ,, | 41 | 120 | 100—1000 | ,, ,, |
| 7 | Supply | Jan. 1913 | 18 | 90 | absent in 50 c.c. | ,, ,, |

A source of contamination sometimes met with as regards deep wells is due to surface water gaining access either by the side of the bore hole down to the deep supply or from the impervious lining being faulty or not extending down far enough. The following is an instructive illustration of this from the point of view of bacteriological examinations. This water supply, which supplies a considerable population, is derived from the mountain limestone and is obtained from a well 31 feet deep with an impervious lining and passing through a thin impervious layer (lower Lias). Usually bacteriological analyses gave satisfactory figures but after very heavy rain unsatisfactory bacteriological results were obtained (*e.g.* Nos. 2, 3 and 4 below). Considerable difficulty was experienced in tracing out the cause of the contamination, but it was ultimately traced to surface water (from the Fuller's earth formation) under certain conditions rising up and passing down by the side of the bore hole to the deep supply. Since steps have been taken to stop this source of contamination the results have been very satisfactory (Nos. 5, 6 and 7) although samples have been purposely taken after heavy rain.

| Sample | Date of examination | Organisms per c.c. at 37° C. | Organisms per c.c. at 21° C. | *B. coli* (per litre) | *Streptococci* (per litre) |
|---|---|---|---|---|---|
| 1 | August 1911 | 20 | 50 | *absent in 10 c.c. | absent in 10 c.c. |
| 2 | Jan. 1, 1912 | 3 | 2500 | 100—1000 | absent in 50 c.c. |
| 3 | ,, 24 ,, | 57 | 170 | 100—1000 | ,, ,, |
| 4 | March 1912 | 220 | 350 | absent in 50 c.c. | 1000—10,000 |
| 5 | June ,, | 2 | 25 | ,, ,, | absent in 50 c.c. |
| 6 | Sept. ,, | 2 | 10 | ,, ,, | ,, ,, |
| 7 | Dec. ,, | 5 | 70 | ,, ,, | ,, ,, |

[* By inadvertence larger quantities were not examined.]

In addition to easily ascertained sources of surface contamination, deep water supplies are sometimes liable to contamination

which is very difficult to locate and for which bacteriological examinations are of the utmost value. Such cases are usually met with in supplies from the limestone or chalk and numerous examples might be quoted.

The following is a good example of intermittent contamination of a deep well in the chalk reported by Richards and Brincker[1]. The well yielded from one to two million gallons per day. The only source of contamination was from surface water rather more than two miles away which was shown to gain access to the deep well water. This was proved by adding a special yellow bacillus to the surface water and tracing it into the well. It was found that the test organism took in one experiment 78·5 hours and in the other 67·5 hours to pass from the swallet hole to the well. Its presence was associated with a marked increase in the bacterial content of the water, together with the finding of *B. coli*, in 10 c.c. or less of the water. The latter organism was absent from the well when no pollution was taking place.

*Shallow wells and Subsoil water.*

Obviously bacteriological findings will vary greatly with this class of waters and very widely varying results are in fact obtained. Sources of bacterial contamination are mainly two, one due to the entrance of bacteria through the mouth or round the sides of the well and the other from bacterial contamination from subsoil water, due to imperfect filtration of bacteria through the soil. In considering bacteriological analyses of surface wells it is always most important to discriminate between these two sources of pollution.

Contamination through the open mouth (in draw wells) or from imperfect fittings and covers round pumps is extremely common and is usually very heavy. Indeed it is very exceptional with open draw wells for their water to show other than gross contamination, and this whether or no the subsoil water itself is contaminated. When such wells are properly covered and their sides are effectively rendered to a depth of 12 ft. or so, no harmful bacterial contamination is sometimes subsequently present, showing that the subsoil water is not itself contaminated.

[1] *Proceedings Roy. Soc. Med., Epidem. Sec.*, 1908, I, p. 191.

The following analyses from a well treated in this way between the two analyses illustrates this point.

| Sample | Organisms per c.c. | | "Excretal" *B. coli* c.c. | | | | Streptococci | | |
|---|---|---|---|---|---|---|---|---|---|
| | 37° C. | 21° C. | 0·1 | 1 | 10 | 40 | 1 | 10 | 40 |
| With defective pump-covering etc. ... | 2350 | over 5000 | − | + | + | + | + | + | + |
| After impervious covering and rendering ... | 9 | 2020 | − | − | − | − | − | − | − |

The bacteriological results[1] obtained with surface wells are variable, but if properly protected from surface contamination and from the entrance of water which has not filtered through at least 12 ft. of soil they will usually show no excretal indicators in 50 c.c. and certainly not in 10 c.c. If "excretal" *B. coli* are present in 10 c.c. or less of a surface well water it is evidence of undesirable and possibly dangerous contamination. Even when only present in 50 c.c. the well and its surroundings should be subjected to very careful examination. The influence of rainfall upon the bacterial content of surface wells is considerable and must be kept in view.

The variable bacterial content of surface wells makes it peculiarly dangerous to pass well waters as satisfactory from the results of a single satisfactory bacteriological analysis. The filtering action of the soil may be for the time satisfactory, but at any moment it may break down and harmful bacteria be washed through into the well water.

For example a recent bacteriological analysis of a well water supplying five houses gave the following results:—Organisms growing at 37° C. and 21° C. = 2 and 15 per c.c. respectively. No *B. coli* or streptococci in 50 c.c.: larger amounts not examined. This well was a shallow one sunk in porous ground and surrounded up to a few yards by heavily manured gardens. It was on inspection obviously liable to pollution, while a chemical analysis disclosed a very high nitrate content (2·0 per 100,000) but low ammonia figures. It is quite clear, as the very good bacteriological analysis showed, that the soil was acting as an efficient filter at the time the samples were collected. The bacteriological data did not, however, disclose that the good quality of the

[1] For numerous analyses and data see Savage, *Journ. of Hygiene*, 1907, VII, p. 477.

water depended upon an efficient soil filtration, and that this might break down at any time and without warning.

### *River Water.*

The terms river and stream cover so many conditions which differ very widely that obviously no possible standards can be forthcoming. Rivers, receiving as they do the washings from the lands they flow through, usually show a high bacteriological content, and as the land so draining is often cultivated, a high content in excretal indicators such as *B. coli* and streptococci. Such contaminated waters obviously cannot be considered as satisfactory sources of supply for drinking unless purification has taken place by sedimentation (storage) or filtration. Their bacterial purity can be judged with great accuracy by their content in the *B. coli* group of organisms.

### *Bacterial Standards.*

From the above considerations it will be evident that no very definite bacteriological standards can be framed, even when each class of water is separately considered. At the same time it is quite possible to frame working guides, as indicated above, and if these are used with reasonable care to obtain most valuable opinions upon any given water supply.

Of the different data available to form such an opinion the *B. coli* group enumeration is by far the most valuable, the other findings being more or less confirmatory. The above considerations set out as a reasonable requirement that "*excretal*" *B. coli* should be absent from 100 c.c. of deep water supplies and from 10 c.c. of surface waters.

Sometimes the organisms isolated are not typical *B. coli*, but differing in the absence of one or more of the characteristic properties of this organism. In the writer's opinion the nearer these lactose-fermenting coli-like bacilli approach typical *B. coli* in their characters, the more nearly are our numerical standards for that organism applicable to them, while if they lack essential characters a proportionately greater number must be present to justify an adverse opinion.

Determinations of the number of streptococci have been made much less frequently than in the case of *B. coli.* As a provisional guide, and without attaching an equal significance to the findings, a standard similar to that for *B. coli* may be employed—*i.e.*, their presence in 100 c.c. or less of deep-well or spring water, or in 10 c.c. or less of surface and shallow-well waters, would justify an adverse opinion as to the purity of the water in question.

On its negative side the streptococcus test is not of great value, and the absence of streptococci, even in a considerable bulk of water, cannot be taken as showing purity or freedom from danger.

Opinion is not united as to the value of *B. enteritidis sporogenes* as an indicator of pollution. It is fairly abundant in sewage and excreta, but it is a spore-bearing organism with prolonged powers of resistance, and therefore, even if it be admitted that its presence indicates pollution, such pollution may have taken place at some long antecedent period, a contamination so old as to be of no significance. Its absence in a large quantity of water is some evidence of purity.

The essential limitation to the value of bacteriological examinations is that they only supply information as to the existing conditions in the water at the time of sampling. They cannot indicate liability to contamination which is not actually taking place. This important limitation must never be lost sight of, and it sets a decidedly restricted value upon the bacteriological examination results of chance samples.

When the bacteriological data discloses distinct evidence of undesirable and potentially harmful contamination it is safe to report adversely upon such a supply, since—unless the sources of contamination can be and are removed—once contaminated always liable to contamination. Even in such cases a condemnatory report comes with much greater weight if more than one sample has been found to be polluted.

It is on its negative side that caution is required. As stated above contamination is often intermittent and chance samples may not disclose it. Opinions on individual samples in such cases should therefore be always statements of fact and not of inference as to the purity of the particular supply.

### *Bacteriological Testing of Filter-Beds.*

The testing of the efficient working of sand filter-beds is entirely a bacteriological matter. All filter-beds should be systematically tested to ascertain the percentage of bacteria removed.

The purification by filtration through sand is only to a small extent mechanical; it is mainly vital. This vital or biological action is due to the formation, which takes place after a few days of working, of a gelatinous layer on the surface of the sand. This is composed partly of suspended matters and partly of bacteria, algæ, and other lowly forms of vegetable life, derived from the water filtered. Such a filter-bed is capable under suitable conditions of removing the vast majority of bacteria from the water. The percentage removed depends upon a number of factors, of which the following are the most important: The rate of filtration, the age of the filter, the depth and size of the sand particles, the kind of filtration—*i.e.*, whether intermittent or continuous—and the nature of the water filtered.

Of these, the first two are the most important. If water is passed rapidly through a filter, the percentage of bacilli removed will diminish. Koch recommended that the rate should not exceed 4 inches per hour. The rate of filtration must however be largely governed by the quality of the water being filtered. The age of the filter is in the main a question of the thickness of the gelatinous layer. The percentage of organisms removed should be 98 or more.

To estimate the bacterial efficiency, or percentage of organisms removed by the filtration, gelatine and agar plates are made from the water before and after filtration, and examined and counted in the ordinary way. Each bed should be separately tested. The rate of filtration and all particulars must be recorded at the same time. The percentage removal of *B. coli* should also be ascertained, and is on the whole a better test of the efficiency of filtration than ordinary bacterial enumerations.

# CHAPTER IV

## SOIL AND SEWAGE

### SOIL

Surface soil contains a vast number of organisms. With increased depth the number rapidly diminishes, and below a metre but few bacteria are to be found in undisturbed soil.

The rapid diminution in the number of bacteria in soil was first clearly established by Fraenkel and has since been confirmed by numerous observers. The following figures will give a good idea of the number of bacteria in soils.

Houston[1], working with soil in the grounds of Morningside Asylum, Edinburgh, on a plot of land which was formerly a vegetable garden, but which had lain untouched for some time, found, as the result of a large number of experiments, that the average number of germs in 1 gramme of soil was on the surface about 1,688,000; at a depth of 1 foot, 1,100,000; 2 feet, 900,000; 3 feet, 174,000; 4 feet, 25,000; 5 feet, 920; and 6 feet, 410. These figures deal only with the numbers which will develop on gelatine media, and do not give any true idea of the total number of bacteria actually present, excluding, as they do, for example, the vast number of nitrifying organisms. They, however, illustrate the rapid decrease with increased soil depth in the number of organisms which grow on gelatine media under aërobic conditions. In Fraenkel's researches anaërobic bacteria were also found to be absent, or relatively absent, in the deeper layers.

The number also varies with the kind of soil, particularly whether virgin or cultivated. Thus, Houston[2], who examined twenty-one samples of surface soil from different sources, found that the virgin sandy soils gave less than 100,000 bacteria per gramme, the other virgin soils about 1,000,000, the garden soils from 1,000,000 to 2,000,000, and two grossly polluted soils,

[1] *Edinburgh Med. Journ.* 1893, XXXVIII, part ii, p. 1122.
[2] *Local Gov. Board Med. Officer's Report*, 1897–8, p. 251.

in one case 26,000,000, and in the other 115,000,000 bacteria per gramme.

The number of spores relative to the total number of bacteria in soil is large, the proportion being often as high as from 1 to 10 to 1 to 3.

The presence of organic matter undoubtedly has some influence upon the number of bacteria in soil.

Reimers[1], examining graveyard soil, found an increased number of bacteria in the vicinity of the coffin, the greatest number being met with some distance above it.

Young[2] examined bacteriologically 23 samples of soil all taken from graveyards, some being from soil which had never been disturbed, others being taken from the vicinity of old interments. He found that the number of bacteria present in soil which had been used for burial exceeded the number in undisturbed soil at similar levels, and that this excess, though apparent at all depths, was most marked in the lower reaches of the soil. Roughly speaking, at the lower depths (8—9 feet) the soil used for burials contained about twelve times as many bacteria as the undisturbed soil and at moderate depths (4—6 feet) about six times as many bacteria.

The bacteriology of soil has been very incompletely worked out, but in addition to the nitrifying bacteria, which are only isolated by special methods, the saprophytic bacteria which are commonly met with are *Cladothrix dichotoma*, *B. proteus vulgaris* and other proteus strains, *B. subtilis*, *B. mycoides*, *B. megatherium*, *B. fluorescens liquefaciens*, *B. fluorescens non-liquefaciens*, *B. arborescens*, and micrococci of different kinds.

The bacteriological examination of soil from the public health point of view is of but limited utility, its chief value being in connection with the contamination of water from surface washings.

### *Pathogenic bacteria in soil.*

Pathogenic bacteria from various sources are constantly gaining access to soil, and accurate knowledge as to their

[1] *Zeitschr. f. Hygiene*, 1889, vol. VII, p. 307.

[2] *Trans. Royal Soc. of Edinburgh*, 1895, vol. XXXVII, p. 759.

vitality and their retention of virulence in such surroundings is of great importance. This is particularly important in connection with the contamination of water supplies. Although numerous investigations have been made it cannot be said that precise information on this subject is available. No doubt the chemical composition and particularly the amount of organic matter in the soil, as well as the number of other bacteria present, play a very important part in the determination of the length of life in soil of those pathogenic bacteria which are not natural inhabitants of the soil.

*B. typhosus.* A number of investigations have been carried out by Sidney Martin, Firth and Horrocks, Rullmann, Dempster, Pfuhl, Savage, Mair, and others, upon the vitality of typhoid bacilli in soil.

Of British investigators working with unsterilized soils Robertson (1898) recovered the bacilli after 300 days, Martin (1896–1901) could only recover the bacilli up to 12 days, Firth and Horrocks (1902) found the bacilli to survive in some cases up to 74 days, Lorrain Smith (1903) up to 21 days the average being 15 days, Savage (1905), in polluted river mud treated bi-weekly with fresh sea water, up to 5 weeks in one case and fairly readily up to two weeks, and Mair (1908) in large numbers for about 20 days and still present after 70 to 80 days.

These results show considerable discrepancies. They show that under favourable conditions the typhoid bacillus will survive for a considerable period in soil, and that the factors influencing its vitality are many and varied, the antagonism of other microbes and the physical conditions of moisture and temperature being the most important.

*B. diphtheriae.* This organism does not appear to have any important relationship to soil, and there is no evidence that infection has resulted from diphtheria bacilli derived from soil. The absence of diphtheria outbreaks spread by water is evidence in the same direction. But little experimental work has been done upon the viability of the diphtheria bacillus in soils, although Germano and others have shown that it may retain its vitality in dust for some time. Leighton has shown that

diphtheria bacilli may remain alive for 18 days in moist warm modelling clay.

*B. pestis.* The Advisory Committee on Plague in India report[1] some experiments on this bacillus in soil. They studied how long floors grossly contaminated with plague bacilli would remain infective for animals, the infectivity being tested by rubbing scrapings into susceptible animals. They found that cow-dung floors remained infective for 48 hours, while floors composed of a mixture of sand and lime allowed to set did not remain infective for over 24 hours.

Gladin claims to have recovered the plague bacillus after two months from unsterilised earth, but Mackie and Winter (quoted in the above report) found the bacillus (with difficulty) up to 96 hours after its introduction, but not subsequently either by culture or animal inoculation.

It would appear that, in general, *B. pestis* dies out rapidly from soil.

*B. anthracis.* As is well known, spores of the anthrax bacillus may live and retain for years their virulence in soil to which they have gained access, and such infected land if used for grazing purposes may serve as a means of infection for very prolonged periods.

A number of anaërobic, pathogenic bacteria are common soil organisms. In particular the bacilli of tetanus and malignant oedema are widely distributed in soil, especially when cultivated. For their characters and general methods of isolation, general text-books on Bacteriology should be consulted.

### *Excretal bacilli in soil.*

The presence and viability of the common excretal bacilli in soil is a matter of considerable public health importance, particularly in relation to water supplies. Soils which have been recently contaminated with organic matter in quantity—for example, by sewage or manure—show evidence of this when bacteriologically examined.

Houston[2], in a prolonged series of experiments, watered

[1] *Journ. of Hygiene*, 1906, vol. VI, No. 4 (extra No.), p. 509.

[2] *Local Govt. Board Med. Officer's Report*, 1900-1, p. 405, and 1901-2, p. 355.

soil with crude sewage and studied the fate of certain sewage organisms—*B. coli*, streptococci, and *B. enteritidis sporogenes*—in the treated soil. While all these organisms were abundantly present in the soils immediately after inoculation, all diminished with time, although their rate of disappearance varied greatly. The majority of the streptococci very rapidly disappeared, the *B. coli* less rapidly, and showed considerable variability, while the spores of *B. enteritidis sporogenes* showed but slight diminution during the period of observation.

Some experiments of the writer[1] upon the self-purification of tipped house and street refuse constituting "made soil," illustrate the gradual replacement of the *B. coli* and other organisms, originally present abundantly in the tipped refuse, by *B. mycoides* and other soil organisms. In these experiments "made soil" (the samples being collected at a depth of 2 feet) was examined, taken from deposits of known ages. In the samples deposited within two years *B. coli* group organisms were abundant, while the numbers of *B. mycoides* and cladothrix were either very few, or these organisms were absent altogether. In material deposited several years previously, on the other hand, no *B. coli* were found, while the soil organisms mentioned were very numerous. The soil organisms had replaced those in the original material. There was a general relationship between the number of *B. coli* and the age since deposit.

The same facts are brought out from the results of the examination of soils from different sources for these sewage and excretal bacteria. Houston, Chick, Savage and others have examined soils for these organisms. In virgin soils typical *B. coli* and streptococci are absent, while spores of *B. enteritidis sporogenes* are either absent, or present in only very small numbers. On the other hand, in cultivated and other contaminated soils all these organisms are present in numbers roughly comparable to the extent and age of the contamination.

The following results obtained by the writer[2] will serve as an illustration of findings likely to be obtained.

[1] *Journal of Sanitary Institute*, 1903, vol. XXIV, p. 442.

[2] *The Bacteriological Examination of Water-Supplies*. H. K. Lewis, London, 1906.

*Table showing results of bacteriological examinations of soil.*

| No. of Sample | Class | Depth | Particulars of Soil | "Excretal" *B. coli* | Coliform Organisms | *B. enteritidis sporogenes* Spores | Streptococci |
|---|---|---|---|---|---|---|---|
| | | | | Number per Gramme | | | |
| 1 | A | Surface | Virgin soil from a wood ... ... ... | Absent in $\frac{1}{5}$ gramme | None found in $\frac{1}{5}$ gramme | Absent in $\frac{1}{10}$ gramme | Absent in $\frac{1}{10}$ gramme |
| 2 | ,, | ,, | ,, ,, } Abundant | ,, | ,, | ,, | ,, |
| 3 | ,, | ,, | ,, ,, } vegetable matter | ,, | ,, | ,, | ,, |
| 4 | ,, | ,, | ,, ,, } | ,, | ,, | ,, | ,, |
| 5 | B | ,, | Grass plot in a garden ... ... ... | ,, | ,, | 100 to 1000 | ,, |
| 6 | ,, | ,, | ,, ,, hospital grounds ... ... | ,, | ,, | Absent in $\frac{1}{10}$ gramme | ,, |
| 7 | ,, | ,, | A ploughed field ... ... ... ... | ,, | ,, | 10 to 100 | ,, |
| 8 | ,, | ,, | Old pasture land ... ... ... ... | ,, | ,, | Absent in $\frac{1}{10}$ gramme | ,, |
| 9 | ,, | ,, | Pasture land near a well ... ... ... | ,, | ,, | 1000 to 10,000 | ,, |
| 10 | C | ,, | Garden soil, manured 4 months previously | ,, | ,, | 10 to 100 | ,, |
| 11 | ,, | ,, | ,, ,, 2 ,, ,, | Not isolated in $\frac{1}{5}$ gramme | In 0·1 gramme | ,, | ,, |
| 12 | ,, | ,, | ,, ,, $3\frac{1}{2}$ ,, ,, | Absent in $\frac{1}{5}$ gramme | Not found in $\frac{1}{5}$ gramme | ,, | ,, |
| 13 | ,, | ,, | Cultivated field ,, 13 ,, ,, | 10 to 100 | ,, | ,, | ,, |
| 14 | ,, | ,, | ,, ,, manured 13 months previously, another part of same field ... | 100 to 1000 | ,, | 100 to 1000 | ,, |
| 15 | ,, | ,, | Garden soil, manured 2 weeks previously | 100 to 1000 | ,, | 10 to 100 | 100 to 1000 |
| 16 | B | 1 foot | Same as No. 5 ... ... ... ... | Absent in $\frac{1}{5}$ gramme | ,, | 10 to 100 | Absent in $\frac{1}{10}$ gramme |
| 17 | ,, | ,, | ,, ,, 6 ... ... ... ... | ,, | ,, | Absent in $\frac{1}{10}$ gramme | ,, |
| 18 | ,, | ,, | ,, ,, 7 ... ... ... ... | ,, | ,, | 10 to 100 | ,, |
| 19 | C | ,, | ,, ,, 10 ... ... ... ... | 10 to 1000 | ,, | ,, | ,, |
| 20 | ,, | ,, | ,, ,, 11 ... ... ... ... | ,, | ,, | ,, | ,, |
| 21 | ,, | ,, | ,, ,, 12 ... ... ... ... | ,, | ,, | ,, | ,, |
| 22 | ,, | ,, | ,, ,, 15 ... ... ... ... | 100 to 1000 | ,, | ,, | ,, |

*The bacteriological examination of soil.*

Soil may be required to be bacteriologically examined for three purposes.

A. To isolate and study the organisms of nitrification and allied processes.

B. To detect the presence of special pathogenic bacteria.

C. To study the degree of pollution with excretal organisms, in relation to water supplies.

A. The organisms concerned with nitrification are present in all soils, and their functions are of vital importance. They are not readily isolated, while their study is chiefly of value for research purposes. Methods of isolation are not therefore included here.

B. The pathogenic bacteria which have to be looked for are those enumerated above, and particularly the bacilli of tetanus, malignant oedema, and occasionally *B. typhosus* and *B. anthracis*. The methods for their examination are in no way special and are conducted on ordinary lines. No detailed consideration is therefore necessary here.

C. *General Soil pollution.* The data which give the most valuable information with regard to recent or remote soil pollution are the following: the total number of aërobic organisms, number of spores present, number of *B. coli*, *B. enteritidis sporogenes*, and streptococci.

In collecting soil for bacteriological examination the depth from which it is obtained is of fundamental importance. If the surface soil is to be examined, scrape up with a sterile spatula, and transfer to a sterile receptacle. To obtain soil from a given depth either a fresh cutting must be made and the soil collected at the required depth, or, preferably, some form of borer may be used. For this purpose Fraenkel's borer is convenient, its chief drawback being that it holds only a small quantity of soil.

If Fraenkel's borer is used, it is advisable to collect at least eight samples from spots about a foot apart, and to mix together

to obtain a representative sample. Also in this way sufficient soil will be obtained for a concurrent chemical examination.

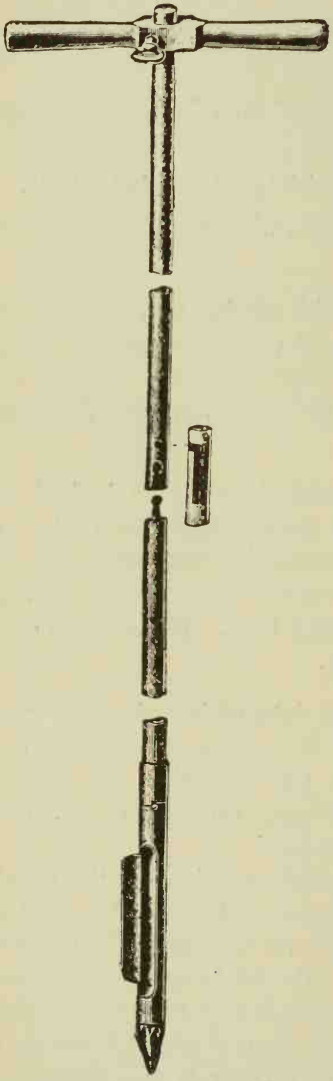

Fig. 7. Fraenkel's Borer.

By means of this borer the exact depth of the soil taken can be ascertained. Owing to its length it cannot be sterilized in the hot-air oven, but it can be conveniently and sufficiently sterilized by pouring in methylated spirit and igniting. After sterilization wrap the lower portion in a sterile cloth and secure with string. This plan is very convenient when a number of samples have to be taken in one day, and at, perhaps, a long distance from the laboratory, since the borer can be resterilized at once before each sample is taken, it being only necessary to carry a bottle of spirit and a number of sterile cloths in a metal box. The soil is removed by a sterile spatula from the interior of the borer to the sterilized tin or other receptacle used for the soil.

The examination should be commenced as soon after collection as possible.

To estimate the total number of bacteria, and for some other steps of the examination, very extensive dilution must be practised. As an example of a convenient method of dilution the following procedure is given: other methods of dilution will readily suggest themselves. It is important to remember that owing to a number of inherent difficulties (such as the difference of coherence of different soils)

numerical estimations are only *relatively* accurate, and in any case the same method should be used throughout for each investigation :

Accurately weigh a small sterile glass-stoppered bottle containing 100 c.c. of sterile water. Quickly weigh in 1 gramme of the soil (previously well mixed together) into the bottle, using a sterile spatula to add the soil. With a little practice 1 gramme can be quickly and sufficiently accurately added. Mix very thoroughly by repeated shaking, if necessary breaking up the soil by a pointed sterile glass rod. Call this solution *Dilution A*. Allow the soil particles to settle, then add 1 c.c. or more, according to the suspected contamination of the soil, to a sterile flask containing 99 c.c. of sterile water. Mix thoroughly and label *Dilution B*. Varying quantities of Dilutions A and B are used for the examination.

To estimate the number of aërobic organisms make gelatine plates from these dilutions. Thus, 0·2, 0·5, 1·0 c.c. of Dilution B are convenient amounts to add to the gelatine tubes. For the number of organisms developing at 37° C. use, in the same way, agar plates.

To estimate the number of spores, present as such, add varying amounts of the dilutions to gelatine tubes. Heat to 80° C. for ten minutes, then plate, incubate, and count in the ordinary way.

For *B. coli* enumerations various fractions of the dilutions are added to tubes of lactose bile salt media. These are incubated at 37° C., and those in which acid and gas are produced are used to inoculate solid media, and the organism isolated exactly in the same way as for the isolation of this bacillus from water.

Streptococci and spores of *B. enteritidis sporogenes* are examined for by methods identical with those used for water.

## SEWAGE

The bacteriological examination of sewage is not, at the present day, a procedure widely practised, although for special purposes it is valuable. It is the only way the potential harmfulness of an effluent can be measured. Chemical standards

and examinations, while of great value, are no guide in regard to the extent to which pathogenic bacteria have been removed by any process of sewage purification.

*Bacterial content of crude sewage.*

As might be anticipated the actual bacterial content of crude sewage varies enormously, since sewages vary so greatly in strength and the extent to which they become mixed with surface water. Speaking generally the number of bacteria present, as shown by the number of colonies on gelatine plates, usually ranges from 1 million to 100 million per c.c. This number will vary from hour to hour according to the strength of the sewage.

The organisms used as indicators of sewage and excretal contamination are all very abundant in sewage, *B. coli* organisms about 100,000, streptococci about 10,000, and spores of *B. enteritidis sporogenes* about 100 to 1000 per c.c.

The number of different kinds of organisms in sewage is very great, and it is probable that many of them occur in all specimens of ordinary sewage, but except for the above organisms their presence has not been ascertained with sufficient constancy, nor has their numerical occurrence been sufficiently investigated to enable them to be used as indicators of sewage pollution. Further investigations in this direction are very desirable.

The organisms of typhoid fever and cholera have never been isolated with certainty from sewage, although they must frequently be added in large numbers when cases of these diseases are present. Anthrax bacilli have been found by Houston in Yeovil sewage, both in the septic tank and in the primary and secondary coke beds, also in the mud of the banks of the river Yeo.

*Effects of sewage treatment upon the bacterial content of sewage.*

The reports of the Royal Commission on Sewage Disposal have made it abundantly clear that while the different processes for treating sewage may effect their immediate purpose of producing a non-putrefying effluent, yet they never yield one which is sterile or anything approaching it. The results obtained

as regards elimination of bacteria vary greatly with the method of purification adopted.

Houston[1] made a careful investigation for the Royal Commission on Sewage Disposal of the biological qualities of the effluents from sewage farms.

Compared with the original sewages, all the effluents exhibited a high percentage degree of purification; but, apart from a reduction in number, they showed no very appreciable biological modification. Houston remarks that sometimes the relative number of spores of *B. enteritidis sporogenes* was reduced, and that there was some evidence, especially in the better class of effluents, of a greater proportionate reduction, as compared with crude sewage, in the number of microbes growing at blood heat, over those growing at 20° C. Also on several occasions he failed to isolate streptococci from the effluent.

In general, however, as Houston very definitely points out (p. 169), "the results conclusively show that the treatment of sewage on land cannot be relied on *materially to modify* the potentially dangerous qualities of crude sewage. The actual number of objectionable microbes persisting in the effluents is too great to allow of much stress being laid on the great percentage reduction effected in the total number of microbes by the land treatment, or to insure any certainty that effluents from land processes are 'relatively safe.'"

Houston[2] also extensively investigated the effluents from biological treatment processes. He sums up the general outcome of the experiments as follows: "The effluents from septic tanks, intermittent contact beds, continuous filtration beds, etc., contain an enormous number of bacteria. In some cases the percentage reduction of microbes in effluent as compared with raw sewage is striking. But as an effluent must be judged by the actual state it is in, and as the number of micro-organisms still remaining is nearly always very large, percentage purification would seem to be of minor importance. In not a few cases the bacteria are practically as numerous in the effluent as in the

[1] *Fourth Report Royal Sewage Commission*, vol. IV, part III.

[2] *Second Report Royal Sewage Commission*, 1902, p. 25.

raw sewage. The different kinds of bacteria and their relative abundance appear to be very much the same in the effluents as in the crude sewage. Thus, as regards undesirable bacteria, the effluents frequently contain nearly as many *B. coli*, proteus-like germs, spores of *B. enteritidis sporogenes*, and streptococci, as crude sewage. In no case, seemingly, has the reduction of these objectionable bacteria been so marked as to be very material from the point of view of the epidemiologist. No definite proof has been furnished that the effluents from bacteria beds are conspicuously more safe in this sense in their possible relation to disease than is crude sewage. Indeed, all the available evidence tends to show that they must be regarded as nearly, if not quite, as dangerous to health as raw sewage.... The inoculation of animals with the effluents from bacterial beds seems to show that they are nearly as pathogenic as crude sewage."

Judged from the bacteriological standpoint the only process which yields a markedly purified effluent is filtration of the sewage through sand. This method is very little used in England, but is employed to some extent in the United States. Extended experiments were carried out by the Massachusetts State Board of Health with sand filters. These experiments showed that 97 to nearly 100 per cent. of the *B. coli* could be removed by these filters, and this whether they were treated with raw or septic sewage.

Although the percentage purification is very great the actual number of *B. coli* remaining is large. Thus the experimental sand filters at Columbus, Ohio, are recorded by Johnson as removing 98·5 per cent. of these bacilli, but 500 to 10,000 *B. coli* per c.c. remained in the effluent.

There does not appear to be much data available to judge as to how far ordinary chemical processes of sewage purification are capable of removing and reducing the bacteria in sewage, but in general it may be said that they are not very effective. Sterilization or partial sterilization of the sewage can be obtained by chemical means, *e.g.* by the use of chlorine evolved in different ways, but these are processes specially applied for this purpose, and are not ordinary chemical methods of purification.

*The fate of pathogenic organisms in sewage.*

The organisms of chief interest are *B. typhosus* and *Sp. cholerae* and a number of investigations have been undertaken to study their viability in sewage.

Klein[1] studied the viability of both organisms in sterile sewage kept at room temperatures. The typhoid bacillus after some preliminary multiplication rapidly diminished in numbers, some bacilli being alive, however, after eight weeks. The cholera vibrio died out at periods varying from the eighth to the twenty-fifth day.

Horrocks found typhoid bacilli alive after 60 days in sterile sewage kept at 16—22° C.

When the sewage is not sterilized the life of the typhoid bacillus is very much shorter, and MacConkey could only recover the bacilli after thirteen days in one series and not after six days in another, while Russell and Fuller found that when this bacillus was exposed directly to the action of sewage bacteria its longevity was greatly diminished, three to five days being the longest time for which the organism could be recovered. The difficulties of isolating typhoid bacilli from crude unsterilized sewage are considerable, even with recent greatly improved technique and bacilli actually present may easily be overlooked and not isolated. After making due allowance for this it is clear that the typhoid bacillus has a hard struggle to live in raw sewage and dies out after a fairly short period.

*The bacteriological examination of sewage and sewage effluents.*

At the present day this is not often required for practical administrative work. The bacteriological examination of sewage effluents is occasionally valuable when the question of pollution of a stream subsequently used for drinking purposes or the possibility of shellfish pollution is under consideration. The general methods are quite similar to those described under water. The chief determinations which will be required are the number of *B. coli* group organisms, the number of spores

[1] *Local Govt. Board Medical Officer's Report*, 1894–5, p. 407.

of *B. enteritidis sporogenes* and the number of streptococci. Compared with water the *B. ent. sporogenes* determination is more valuable and that for streptococci less valuable.

Great initial dilution of the sample is required before the different fractions are removed for examination, the dilutions to be used depending upon the degree of probable dilution of the effluent. Dilutions varying from 0·1 to 0·000001 c.c. should be examined from unknown samples in routine cases.

It must be remembered that the bacterial content of both sewage and sewage effluent samples varies from hour to hour. To get representative results samples would have to be taken every hour and in amount proportional to the flow, kept in ice until the end of the 24 hours, pooled and a representative mixed sample taken for bacteriological examination. It is only in very rare cases that it is worth while taking all this trouble. As a rule all that is necessary is to show that samples of effluent taken at different times contain these indicator organisms in large numbers, often not materially reduced from their numerical presence in the crude sewage, and that therefore it is a reliable deduction that the sewage effluent in question is not safe to discharge into any stream which itself may ultimately be used for drinking purposes or which will wash food material—watercress, shellfish, etc.—to be used for human food.

## CHAPTER V

### SHELLFISH

It has now been conclusively proved that shellfish may and do convey disease to man, typhoid fever in particular, but probably also other diseases. This fact has directed considerable attention to the bacteriology of shellfish and to their bacteriological examination to detect and measure the extent of their bacterial contamination.

This bacteriological investigation has been directed along three lines:

A. To enable bacteriology to be used to judge to what extent any given batch of shellfish is dangerous to health.

B. To enable judgment to be given as to the safety of any given shellfish layings.

C. To study the conditions under which dangerously contaminated shellfish can be rendered free from their bacteriological pollution and made safe for human consumption.

The shellfish chiefly concerned in the bacteriological transmission of disease are oysters, mussels and cockles.

In connection with this subject the general bacteriology of sea water and the sea water of estuaries is of considerable importance.

*The bacteriology of oysters in relation to sewage pollution.*

The general bacterial content is of but little significance and the only recorded data of importance refer to the extent to which oysters contain the organisms used as bacterial indicators of sewage. The following examples from Houston's work[1] give an excellent idea of this as regards oysters from different sources.

(*a*) *Deep sea oysters.* Samples were examined from a number of different sources. A very few organisms partially resembling *B. coli* were isolated. Houston concludes: "Judging from these experiments as a whole, the conclusion seems inevitable that in deep sea oysters derived from deep sea water remote from sewage pollution *B. coli* and coli-like microbes and also the spores of *B. enteritidis sporogenes* are either absent or, at all events, seldom detectable. The same is true of the surface water over such oysters."

"*B. coli* and *B. enteritidis sporogenes* seemingly form no essential part of the bacterial flora of pure sea water, and they have no part in the economy of the oyster."

(*b*) *Oysters from estuaries not exposed to serious sewage contamination.* The Helford River traverses a sparsely-populated

[1] *Royal Commission on Sewage Disposal, Fourth Report*, 1904, vol. III.

district, and ranks as one of the purest localities in England for the growth and fattening of oysters. Houston says it is a river which "on topographical grounds, has been, and would still be considered eminently well suited for the breeding, growth and fattening of oysters for market."

The following results were obtained by Houston with oysters from this source, while the sea water results are also included for comparative purposes.

*Helford sea water.*

| *B. coli* or coli-like microbes in | | | *B. enteritidis sporogenes* |
|---|---|---|---|
| 100 c.c. | 10 c.c. | 1 c.c. | 10 c.c. |
| Positive result | Positive result | Positive result | Negative result |
| 28 % | 40 % | 32 % | 100 % |

*Helford oysters.*

*B. coli* test.

1 out of 25 (4 %) contained 1 *B. coli* or coli-like microbes per oyster.
5 ,, (20 %) ,, 10 ,, ,, ,, ,,
16 ,, (64 %) ,, 100 ,, ,, ,, ,,
3 ,, (12 %) ,, 1000 ,, ,, ,, ,,

*B. enteritidis sporogenes* test.

16 out of 25 (64 %) contained less than 10 spores per oyster.
8 ,, 25 (32 %) contained 10 but less than 100 spores per oyster.
1 ,, 25 (4 %) ,, 100 ,, 1000 ,, ,,

(*c*) *Oysters from estuaries seriously contaminated with sewage.* The Penryn River is polluted and its oyster layings lie under the ban of suspicion. "The Penryn River, on topographical and epidemiological grounds, would be regarded with great suspicion, if not condemned."

The following results were obtained by Houston.

*Penryn water.*

| *B. coli* or coli-like microbes in | | | | *B. enteritidis sporogenes* | |
|---|---|---|---|---|---|
| 10 c.c. | 1 c.c. | 0·1 c.c. | 0·01 c.c. | 10 c.c. | 10 c.c. |
| Positive result | Positive result | Positive result | Positive result | Positive result | Negative result |
| 16 % | 36 % | 44 % | 4 % | 56 % | 44 % |

*Penryn oysters.*

*B. coli* test.

1 out of 25 (4 %) contained 100 *B. coli* or coli-like microbes per oyster.
13 ,, (52 %) ,, 1000 ,, ,, ,, ,,
11 ,, (44 %) ,, 10,000 ,, ,, ,, ,,

*B. enteritidis sporogenes* test.

3 out of 25 (12 %) contained 10 spores of *B. enteritidis sporogenes* per oyster.
20 ,, (80 %) ,, 100 ,, ,, ,, ,,
2 ,, (8 %) ,, 1000 ,, ,, ,, ,,

These three typical groups of results show that *B. coli* and *B. enteritidis sporogenes* are present in oysters in amounts roughly proportionate to the extent of their pollution with sewage. In the same way as for water supplies it is not the presence of these organisms which has to be considered but their relative abundance. Even oysters from reasonably pure sources will frequently show some *B. coli* and it is only deep sea oysters which are quite free. These general conclusions are in accord with the work of Klein, McWeeney, Clark and Gage, etc.

As regards standards for comparative purposes Houston[1] has given the very useful classification contained in the table on p. 78.

*The bacteriology of mussels in relation to sewage pollution.*

Very few data seem to be available as to the bacteriological content of mussels from perfectly pure surroundings. Some of the earlier workers (*e.g.* Herdman and Boyce) found *B. coli* organisms to be usually absent but these results were probably due to incomplete examinations and are not in accord with later experience.

Johnstone[2] from extensive work states: "my own experience has been that no sample of ten or more mussels can be examined without finding *Bacillus coli*, or at least some organisms resembling this form." Even with mussels examined well away from considerable sewage contamination (such as at some beds in Morecambe Bay) *B. coli* were numerous. In the case of ten mussels from this source the number of intestinal bacteria isolated from about 0·2 c.c. of the stomach juices varied from 3 to 65, average 24·2.

[1] *Journ. of Hygiene*, 1904, vol. IV, p. 182.
[2] *Journ. of Hygiene*, 1910, vol. IX, p. 412.

*Bacteriological Grouping of Oysters into Classes, with Tentative Standards for Comparative Purposes.*

| Class* | Standard based on numerical abundance of *B. coli* (or non-liquefying, gas-forming coli-like microbes) in the whole contents of the oyster-shell (*i.e.* liquor, body and interior juices of the oyster) | Numerical standard confirmed or modified according to response of the coli-like microbes in pure culture to certain well-known biological tests | Provisional bacteriological conclusions confirmed or modified by topographical observations | Provisional bacteriological and topographical conclusions confirmed or modified by epidemiological and administrative considerations |
|---|---|---|---|---|
| I<br>An oyster showing no evidence (bacteriologically) of objectionable contamination | No *B. coli* | For example:—<br>(1) *Neutral-red broth test.* Greenish-yellow fluorescence (48 hours at 37° C.)<br>(2) *Lactose peptone test.* Gas and acid production (48 hours at 37° C.)<br>(3) *Indol test.* Indol in broth cultures (5 days at 37° C.)<br>(4) *Litmus milk test.* Acid clotting of milk (5 days at 37° C.)<br>Of course, the more tests applied, the better, but the above are all known tests of value | For example:—<br>Dilution: set of the tides; prevailing winds; float experiments; time interval; distance, etc., etc. | For example:—<br>Questions of practicability; whether the contaminating material is likely to have a high or a low enteric morbific value; past epidemiological experience in circumstances broadly parallel, etc., etc. |
| II<br>An oyster showing appreciable, although slight, evidence (bacteriologically) of objectionable contamination | 1 *B. coli* per oyster† | | | |
| III<br>An oyster showing definite signs (bacteriologically) of pollution, etc., therefore, possibly to be viewed with some degree of suspicion | 10 *B. coli* per oyster | | | |
| IV (Stringent standard)<br>An oyster showing such obvious signs (bacteriologically) of pollution as to be condemned on a stringent standard | 100 *B. coli* per oyster | | | |
| V (Lenient standard)<br>An oyster showing such unmistakeable evidence (bacteriologically) of pollution as to be condemned on the basis of results‡ | 1000 *B. coli* per oyster | | | |
| VI<br>An oyster showing such *gross* evidence (bacteriologically) of contamination as to be outside the pale of recognition | 10,000 *B. coli* per oyster | | | |

* It must, of course, be definitely understood that it cannot be said either that oysters of Class II or even of Class I are necessarily always safe, or that oysters of Classes III—VI have a definite "disease value."

† To obtain approximate results *per c.c.* of oyster, divide the foregoing figures by ten.

‡ This does not mean administrative, practical or legislative condemnation, but only that the evidence of pollution is sufficiently defined to merit objection from the bacteriologist's point of view.

Buchan[1] examined 25 samples of raw mussels from 15 known sources coming into Birmingham. The bacteriological examinations were not associated with any investigation into the actual conditions at the layings nor are such important particulars as the conditions and the time of storage set out. These omissions make the results of no value as a basis of comparison with topographical conditions but they are useful as giving some idea of the bacterial content of mussels as often put upon the market. The method used enabled the results to be calculated as per mussel.

The organisms growing upon gelatine plates averaged about 1000 million per mussel with variations of from 7 million to over 7000 million. On agar at blood heat the average was 182 million with variations of from 2 million to 2400 million.

As regards *B. coli* group organisms the results per mussel were:

| | | | | | |
|---|---|---|---|---|---|
| 1—10 | *B. coli* organisms | in | 2 | batches. |
| 10—100 | „ | „ | „ 7 | „ |
| 100—1000 | „ | „ | „ 10 | „ |
| 1000—10,000 | „ | „ | „ 3 | „ |
| 10,000—100,000 | „ | „ | „ 2 | „ |
| over 100,000 | „ | „ | „ 1 | batch. |

*B. enteritidis sporogenes* (milk change).

Absent in 1 sample.

| | | | | |
|---|---|---|---|---|
| 10—100 | spores per mussel | in | 13 | samples. |
| 100—1000 | „ | „ | 10 | „ |
| 1000—10,000 | „ | „ | 1 | sample. |

*Streptococci.* Not found in five. In the others present, 10—100 per mussel in three, 100—1000 in five, 1000—10,000 in six, and 10,000—100,000 in six.

*The bacteriology of cockles in relation to sewage pollution.*

No definite and extensive comparative work appears to have been carried out in regard to the bacteriology of cockles in relation to topographical conditions and degrees of sewage pollution. From the different analytical results recorded by Klein and others,

[1] *Report to Birmingham City Council*, 1908.

it is evident that cockles are frequently extensively polluted and show sewage organisms in very large numbers. A number of isolated examinations are recorded by Klein[1]. With cockles taken from extensively polluted layings in the river Orwell, the *B. coli* in the stomachs of the cockles varied from 4000—5000 per cockle. On the other hand, with a batch of cockles taken from layings far away from sewage contamination, only one of the cockles contained a *B. coli*-like organism and all were free from true *B. coli*, streptococci or spores of *B. enteritidis sporogenes*.

The writer has examined a large number of cockles taken from various sources. Although some of them were personally collected from sources well away from any considerable degree of sewage contamination they all contained *B. coli*. The numbers present were, however, roughly in proportion to the extent of the sewage contamination known to be present.

*The purification and self-purification of shellfish.*

The investigations of Klein, Herdman, Herdman and Boyce have shown that when oysters are artificially inoculated by being placed in water containing very numerous typhoid bacilli, these bacilli can be recovered from the oysters for from 10 to 18 days subsequently, the actual period varying in the different experiments. Herdman and Boyce[2] found that the bacilli did not increase in the body or tissues of the oyster. When infected oysters were washed in a stream of clean sea water there was always a great diminution or total disappearance of the typhoid bacillus in from one to seven days.

Klein[3] in a later series of experiments showed that oysters infected with very large numbers of typhoid bacilli cleaned themselves, if placed in clear sea water, within a short period. Klein's experiments showed that after eleven days the number of typhoid bacilli were reduced enormously since they could not be found when one-eighth part of an oyster was examined. These results are not conclusive in regard to the *total* disappearance of the typhoid bacilli.

[1] *Oysters and Shell-fish, Report of the Fishmongers' Company*, 1902–9.

[2] *Thompson-Yates Laboratory Reports*, 1898–9, vol. II.

[3] *Experiments on Vitality of B. typhosus in Shell-fish*, 1905.

When cockles were infected with typhoid bacilli, by infecting the sea water in which they were placed with this bacillus, Klein, in a single experiment, found that after ten days the cockles were still heavily infected with typhoid bacilli. In the same way with mussels the cleansing was only relative, in the few experiments carried out.

Johnstone[1] carried out some experiments on the self-purification of mussels. Mussels were used taken from an undoubtedly polluted source and containing, on an average, 1900 intestinal bacteria per shellfish. They were then placed in wooden boxes in sea water which while not unpolluted was reasonably clean. After four complete days some of the mussels were examined and the results showed that the intestinal bacteria had been reduced to about 150 per shellfish (93 per cent. reduction). They were left for a further three days but no, or very slight, reduction in the number of bacilli occurred.

Carnworth[2] also carried out two experiments with mussels. The numbers of *B. coli* were reduced 100—500-fold after four days' stay in pure sea water and a longer stay still further reduced their numbers. A 99 per cent. reduction was met with within a few days.

Houston[3] took 250 oysters from polluted layings and relaid them in a locality free from sewage pollution. The experiment lasted for only 26 days and during this time the oysters showed no material diminution in the number of *B. coli* organisms.

*Bacteriological examination of sea water and tidal mud.*

In connection with shellfish examinations it is always of value to bacteriologically examine the sea water over the layings and, in particular, to examine the tidal mud of the estuary in which they are contained and from their neighbourhood.

The writer[4] in 1904 collected and examined a considerable number of samples of mud from a tidal estuary, at the same time making a careful topographical examination of the sources of

[1] *Journ. of Hygiene*, 1910, vol. IX, p. 412.
[2] *Brit. Med. Jour.* 1909, II, p. 695.
[3] *Royal Commission on Sewage Disposal*, *4th Report*, 1904, vol. III.
[4] *Journ. of Hygiene*, 1905, vol. V, p. 146.

contamination. In the creek of the estuary was a very large oyster fattening ground.

The polluted muds showed 10,000 to 100,000 *B. coli* per gramme of mud where most polluted, decreasing very regularly to 10 or less than 10 per gramme in the unpolluted creeks. The number of streptococci showed similar and gradual diminutions from 100 to 10,000 per gramme to less than 10 per gramme.

The writer concluded that mud samples yield more reliable bacteriological evidence of the degree of contamination of a tidal river than either water or oyster samples. The latter only indicate immediate and actually present pollution while mud samples show evidence of past contamination. If the muds show a relatively high purity they point to a safe fattening area for oysters.

Data as to the intestinal bacteria in sea water over oysters is given on pages 75 to 77.

*The bacteriological examination of shellfish.*

A. *Methods of collection and transmission.* Care must be taken to collect a representative sample and precise local particulars must be recorded. Shellfish should be collected from different parts of the area as some parts may be much more liable to contamination than others. The relationship of the beds to the tide (if, as is usual, they are in tidal waters) must be noted, particularly the extent to which the beds are covered or uncovered by the tide. The neighbourhood and distances from all sources of contamination, particularly sewer outfalls, must be carefully recorded.

The samples must be transmitted for examination without delay and under conditions which do not allow of contamination in transit.

In the examination of consignments of shellfish collected and brought into the market it is very desirable to obtain all possible particulars in regard to the time since collection and the intermediate conditions of storage. These particulars may not be necessary for judgment as to the contamination or otherwise of the particular samples, but they are necessary if the examination is to be used in any way as a guide to the condition of the beds themselves.

B. *Methods of sampling and dilution.* These differ slightly according to the shellfish.

*Oysters.* The most thorough method for the bacteriological examination of oysters is that used by Houston for his work for the Royal Sewage Disposal Commission. The procedure is briefly as follows:

1. The outside of the oyster-shells are well scrubbed with soap and water, and cleaned as thoroughly as possible in running tap-water, finally with sterile water.

2. The hands of the investigator are thoroughly cleaned, washed in 1 in 1000 corrosive sublimate solution, and finally with sterile water.

3. The oysters are opened by a sterile knife held in position by a sterile cloth, and with the concave shell underneath. Great care must be taken to avoid any loss of the liquor. The liquor in the shell is poured into a sterile 1000 c.c. cylinder, and the oyster and oyster liquor are added after the oyster has been cut into small pieces by sterile scissors.

4. Ten oysters are to be treated as above in each experiment.

5. The volume of oyster + oyster liquor is read off, and usually varies between 80 and 120 c.c. For qualitative work 100 c.c. may therefore be taken as a fair average of the total shell contents of ten oysters.

Sterile water is then poured into the cylinder up to the 1000 c.c. mark, and the whole well stirred with a sterile rod. Each 100 c.c. of this liquor may be considered to contain the bacteria in one oyster.

10. Various amounts and fractions of this liquor are used for the examination for *B. coli*, *B. enteritidis sporogenes*, and for streptococci.

*Mussels.* The most satisfactory method is one similar to the above, the whole mussel being used. The mussels are thoroughly cleaned, also the hands of the investigator, as for oyster examinations, while the procedure is quite similar after the shellfish are opened.

Buchan found that a mussel of average size has a bulk of about 15 c.c. but considerable variations occur with individual mussels.

When the only opinion required is as to the relative purity of mussels from different sources, and not a quantitative enumeration, the following method of Johnstone (*loc. cit.*) may be found useful.

The mussels are washed under the tap and opened so that the adductor muscles of the shell, the pedal muscles, and the muscles of the mantle, are alone cut through. The soft parts of the mollusc are retained in the right-hand valve of the shell. Sterilized knives are prepared, two for each shellfish. A slit is made in the body of the animal immediately over the stomach and through the dark green "digestive gland"—really an extension of the lumen of the stomach—and a small quantity of the stomach juices is withdrawn by a sterile pipette with rubber teat (previously made in batches and drawn out to deliver approximately the same volume of liquid) and distributed over the surface of an L.B.A. plate. The volume of fluid taken amounts to about 0·1 c.c. and usually a mussel contains enough to make two or three separate inoculations. In this way a comparative estimate of the number of *B. coli* group organisms is obtained.

*Cockles.* These small shellfish should be treated in the same way as oysters. A sample of cockles consisting of ten animals should be made up to 100 c.c. One c.c. of the cockle mixture then contains 0·1 animal.

C. *Bacteriological procedures.* The number of organisms developing upon gelatine and agar plates can be obtained in the ordinary way but the enumeration is of no value and is scarcely worth doing.

The determinations of value are those for the ordinary excretal indicators, *B. coli* group organisms, spores of *B. enteritidis sporogenes* and, to a less extent, streptococci.

For *B. coli* Houston used 0·0001, 0·001, 0·01, 0·1, 1, 10 and 100 c.c. the amounts being added to bile salt broth, while for *B. enteritidis sporogenes* the two extremes were omitted.

The actual isolation is carried out in the ordinary way. As mentioned above for rough comparative purposes Johnstone used direct plating of definite amount of shellfish juices upon L.B.A. media.

# CHAPTER VI

## MILK

The bacteriological examination of milk even up to a few years ago could not be said to be very satisfactory, and indeed, apart from tuberculosis, very little was systematically done, while in regard to the procedures that were carried out there was neither uniformity of method nor consensus of opinion as to what was desirable. Even at the present time it is by no means on a uniform or satisfactory basis.

The bacteriological examination of milk may be utilised to give information in the three following directions:

1. To measure the degree of contamination of the milk from faecal and other sources and its general bacterial content.

2. To ascertain the presence or absence of definite disease-producing organisms, *e.g. B. diphtheriae*, *B. tuberculosis*, *B. typhosus*.

3. To obtain evidence as to the healthiness of the milk-producing apparatus of the cows which supply the milk.

The procedures to be used will obviously require to be varied according to the purposes of the examination. The different methods are considered in detail in the following sections.

### *Collection of Samples.*

Care in collection is necessary to obtain samples which are really representative of the milk to be examined. Ordinary samples of mixed milk may be collected at the byres or in course of transit or delivery. The cream and the sediment are

the richest in bacteria, so that it is necessary to well mix the milk before a sample is withdrawn.

In the collection of samples from individual cows care must be taken to avoid outside contamination. The cows' udders and teats must be washed carefully, and the milker must wash and disinfect his hands. The milk is milked direct into the bottle, the stopper being held by its free end by a second person to avoid contamination, and inserted immediately after the sample

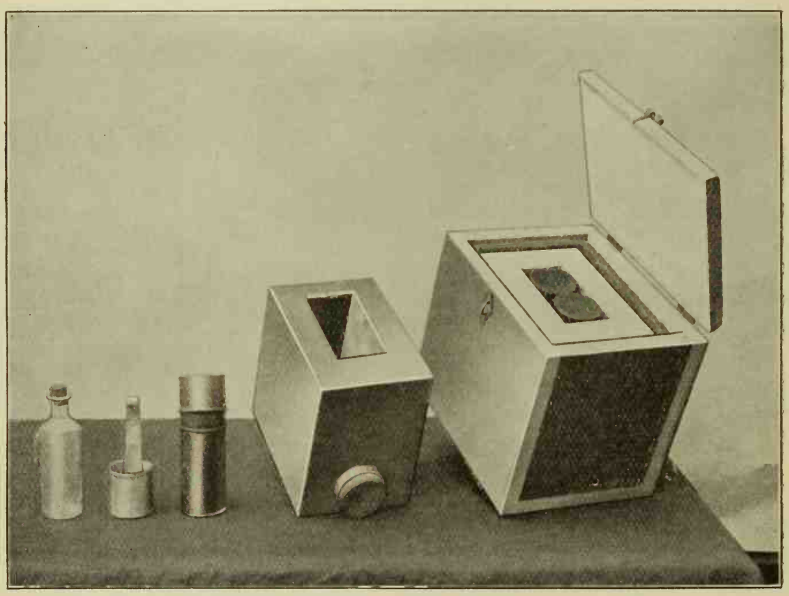

Fig. 8. Collecting bottle and ice box.

has been collected. The bottle should not have too narrow a mouth. In some cases it may be sufficient to collect a mixed sample from the four quarters, and then care should be taken to obtain as nearly as possible equal quantities of milk from each teat. In other cases it may be necessary to collect a separate sample from each quarter. In general the fore milk should be rejected and middle milk sampled. In rare instances it may be necessary to collect fore, middle, and end milk samples from one special quarter or from each of them.

The milk should be collected in sterile bottles with accurately

fitting glass stoppers. For the ordinary examination of mixed milk a sample of about a pint is a convenient amount to obtain, but for most purposes a much smaller quantity is sufficient—particularly for samples from individual cows.

To collect the sample the simple and efficient apparatus described by Delépine (Fig. 8) may be used. It consists of a metal case containing a 7 or 8 oz. bottle and a milk-scoop. All the parts are thoroughly sterilized in the laboratory before being sent out, and the sterilized case is opened only at the time when the sample is taken. The sterilized scoop is used to remove the milk from the cans or other vessels.

If the samples have to be transmitted any distance, or cannot be examined within an hour or two, they must be packed in ice.

The ice-box used by Delépine (Fig. 8), which is similar to the apparatus the writer uses for bacteriological samples of water, is very convenient. The size the writer uses is made to just hold four 2 oz. bottles and their tins, but larger bottles are more convenient for milk samples. The glass-stoppered bottles are sterilized in their tins, into which they just fit, being prevented from moving by layers of asbestos cardboard above and below.

Some alteration in the bacterial content takes place at 0° C. or a few degrees above this temperature but it is not marked for several days and there is no material alteration or error of judgment in examining samples kept iced for not more than 12 to 20 hours.

### *Particulars to record with the Samples.*

Certain particulars must be carefully recorded, and should include:

(*a*) Date and time of sampling.

(*b*) Identification details as to farm or person from whom the milk is obtained.

(*c*) If mixed milk or from individual cows.

(*d*) If mixed milk, whether collected from byre, in transit, in shop, etc., or as delivered.

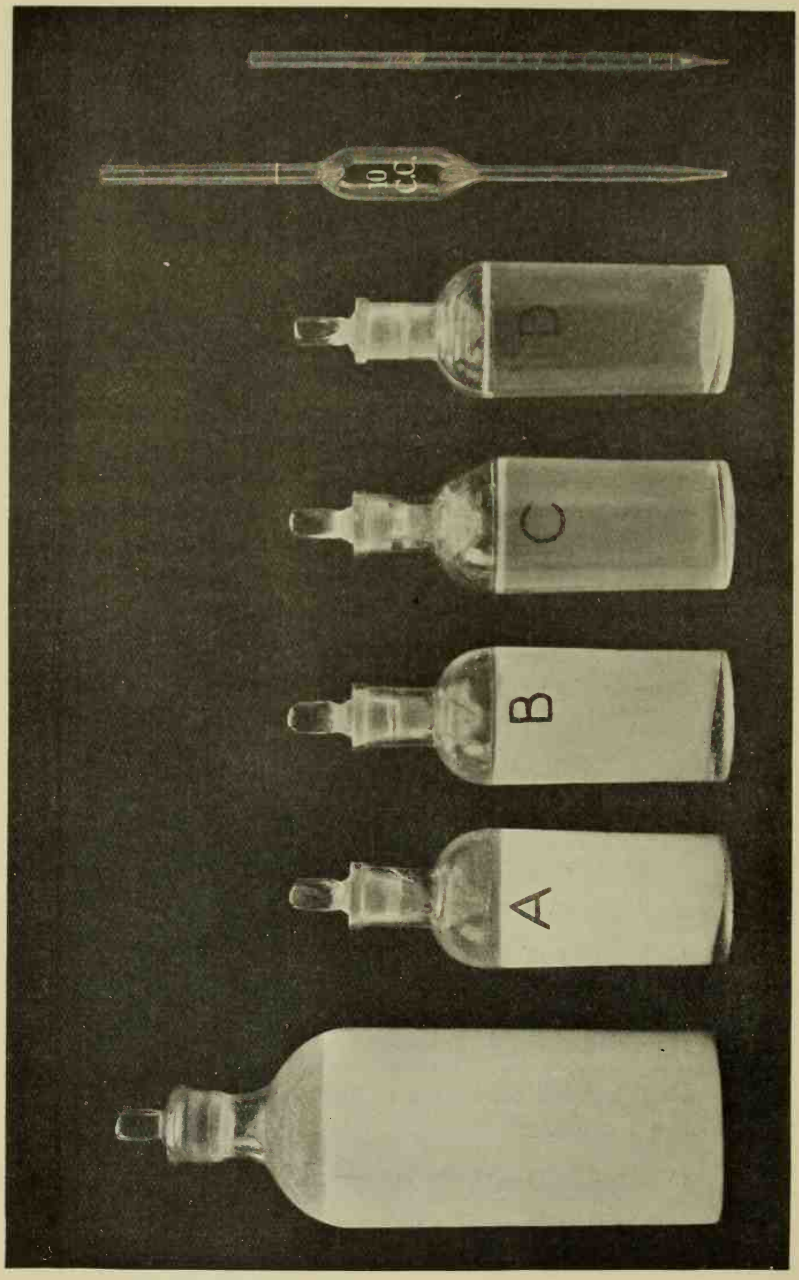


Fig. 9. Dilution of milk samples.

(*e*) If collected at the byre, the number of cows from which obtained should be given, and whether the milk has been strained or not, and if so the strainer used.

(*f*) If from individual cows, particulars of the quarter or quarters from which obtained, and if fore, middle, or end milk.

It is useful to take and record the temperature of the milk.

### *Dilution of the Sample.*

In the bacteriological examination of milk the proper dilution of the sample is of the utmost importance if accuracy is to be obtained. It is important that the milk should be thoroughly well shaken before dilution, and that the dilutions should be well mixed.

The following is the most convenient method of dilution. A large number of glass-stoppered bottles of about 120 c.c. capacity are used, each containing 90 c.c. of sterile tap-water (Fig. 9). Sterile one-mark 10 c.c. pipettes are conveniently used to add the milk, and these should be made short for convenience of sterilization. After thorough shaking 10 c.c. of the milk is removed and added to a 90 c.c. dilution bottle (Dilution A). After well mixing 10 c.c. Dilution A is added to a second 90 c.c. bottle (Dilution B). In the same way Dilution C is made from B and Dilution D from C. Each dilution, of course, represents one-tenth dilution of the one immediately above it in series.

Small 1 c.c. pipettes graduated in tenths of a c.c. are used to add fractions of the different dilutions to the requisite media.

Dilution flasks or bottles containing 99 c.c. or 9 c.c. are often used, and are recommended by the American Committee on Standard Methods of Bacterial Milk Analysis[1], but the writer is of opinion that the addition of only 1 c.c. of milk for the primary dilutions is unreliable and leads to error. By adding as much as 10 c.c. errors of measurement are reduced to a minimum, while the use of glass-stoppered bottles enables the dilutions to be very thoroughly mixed.

[1] *American Journ. of Public Hygiene*, 1910, xx, p. 315.

### *Sources of bacteria in milk*[1].

Milk as *secreted* is a sterile fluid, but during every stage from the udder to the consumer, contamination is possible, and under many of the present-day conditions is invited. In consequence, milk as vended may, and frequently does, contain very many thousands of bacteria. The sources by which bacteria gain access to milk can be grouped as follows:

1. Intra-mammary.
2. Introduced during the milking operation.
3. From ordinary milk utensils.
4. From the use of special milk apparatus.
5. Contamination in transit.
6. Contamination upon the purveyors' or consumers' premises.

1. *Intra-mammary Contamination.* The view generally accepted up to a few years ago was that the interior of the udder contained no bacteria, and that milk, if not quite sterile when it left the teats, yet contained but a few bacteria derived from the teat ducts themselves. This opinion was largely based upon the fact that with a sterile tube it was sometimes possible to obtain sterile milk from the udder. More recent investigations have, however, shown that this is not in accordance with the facts and that undoubtedly the teat duct, the milk cistern and the larger ducts usually contain bacteria whose primary origin is outside the teat. Some selective action has however taken place, the organisms found being almost invariably staphylococci, streptococci and other forms of micrococci.

2. *Introduced during the Milking Operation.* The bacteria introduced during milking are derived from three sources, (*a*) the coat, udder and teats of the cows; (*b*) dust from the milking shed and the clothes of the milker, and (*c*) derived from the hands of the milker.

[1] For a more detailed account of the bacteria in milk see Savage, *Milk and the Public Health*, Macmillan and Co. 1912.

All these sources add bacteria to the milk, the first probably being responsible for the greatest contamination.

3. *Bacteria introduced from Milk Vessels.* When imperfectly cleaned these are a source of great bacterial contamination to the milk.

4. *From Special Milk Apparatus.* The commonest apparatus used, not strictly appertaining to milking, is a milk-strainer. It is an almost universal practice of the cow-keeper to strain the milk, usually immediately after milking. If not kept absolutely clean milk strainers may actually add bacteria to the milk.

Milk-coolers are sometimes a source of bacteria in milk. They may be placed in dusty places so that the milk passing over them, exposing as it does a large surface to the air, takes up a good many bacteria. Also they may not be kept perfectly clean, traces of milk being left in which bacteria multiply enormously and are subsequently added to the milk. A third but much less common way by which coolers may contaminate the milk, is by being leaky, the water being added in small quantity to the milk.

In the same way milk-separators usually add bacteria to the milk passing through them as estimated by numerical counts. This increase is however largely apparent and due to the breaking up of clumps of bacteria.

5. *Contamination in Transit.* It is an extremely difficult thing to measure the amount of bacterial contamination of milk which takes place in its transit from cowshed to milk purveyor or consumer. The increase in the number of bacteria added from outside sources is obscured by the increase due to the multiplication of the bacteria already in the milk.

The addition of bacteria from outside in transit is probably trivial compared with the bacterial additions at the cowshed and in the consumer's house.

6. *Bacteria added on the Purveyor's or Consumer's Premises.* Obviously the number of bacteria added must vary enormously with the condition of the premises and the cleanliness practised upon the premises.

*Procedures to determine the Manurial and General Bacterial Contamination of Milk.*

The above considerations show that the sources whereby bacteria gain access to milk are very numerous and diverse, while they are operative very unequally, according as cleanliness conditions are or are not practised.

To gauge this general bacterial contamination, the following estimations have to be considered.

A. *Estimation of the Number of Bacteria.*

The estimation of the total number of bacteria in milk is impossible, and it is necessary to select an arbitrary basis for enumeration. There are no known nutrient media, and no known conditions of growth which will allow *all* the bacteria in milk to develop. All we can say is that some media and some conditions are more favourable to the growth of a larger number of the bacteria in milk than are others, and by their employment a higher count is obtained than by the use of less favourable media and conditions. The bacterial count being relative, not absolute, any enumeration method for the bacteria in milk should aim not so much at obtaining the largest count, but one which shows best the evidences of manurial pollution. Some investigators use nutrient gelatine, others nutrient agar, others whey agar, etc. In the writer's opinion nutrient agar (of +1 reaction), with the plates incubated for forty-two to forty-six hours at 37° C., is the most convenient and satisfactory, but gelatine plates grown for three days at 20° to 22° C. are used by many.

With unknown samples an extended series of dilutions will have to be employed for plating in order not to have overcrowded plates. A rough idea of the number of bacteria in a given milk sample, and so a guide obtained as to the dilutions to plate, can be ascertained from an examination of the stained centrifugalized deposit.

In milk examinations involving bacterial estimations, the presence or absence of preservatives should be ascertained, as if present they will have an inhibitory or retarding action on the number of bacteria, and a stale milk so treated may show a low bacterial figure.

### B. *Estimation of the Number of Bacillus coli and allied Organisms.*

The general principles and most suitable methods have been discussed in Chapters I and II so that only any special modifications for milk samples require to be considered.

The methods to use differ in no way from those employed for water samples except in regard to the dilutions to add to the tubes of lactose bile salt broth.

Definite fractions of milk are added by pipette, the amounts depending upon the suspected degree of pollution of the milk. A usual procedure is to add 1·0, 0·1, 0·01 c.c. if the sample is byre milk, and if it is ordinary vended milk to add, in addition, 0·001, 0·0001, 0·00001, 0·000001 c.c. and occasionally even smaller fractions. The dilutions are obtained as described above.

The *B. coli* group organism is isolated from the smallest amount giving gas and acid after incubation at 37° C. for two days.

These dilutions are widely spaced, and consequently the number of *B. coli* present can only be enumerated between rather wide limits. The writer prefers therefore to add several equal amounts to lactose bile salt broth tubes. Thus for samples collected at the byre he adds 1 c.c. to each of five broth tubes and 0·1 c.c. to two others, while for ordinary vended milk samples greater dilutions are used, *i.e.* 0·0001 c.c. four, 0·001 c.c. four, 0·01 one, and 0·1 one.

By this procedure a much closer estimate of the number of *B. coli* can be obtained than by the ordinary methods and with but very little more expenditure of time and material.

### C. *Estimation of the Number of B. enteritidis sporogenes Spores.*

The milk itself is directly incubated. The usual quantities of milk to examine are 1, 10, and 20 c.c., the smallest amount being added to a tube of freshly sterilized whole milk, while the other quantities are placed in empty sterile test tubes. The milk tubes are heated in a water-bath to 80° C., and kept at that

temperature for ten minutes, then cooled and incubated anaërobically as described in Chapter II.

The amounts given above are too wide apart to yield a satisfactory estimation, and the following method is advocated by the writer[1]: quite small, narrow (4 by $\frac{1}{4}$ inch), sterile empty test tubes are used in batches of ten for each estimation, 20 c.c. of milk is employed for each test, 2 c.c. being added by sterile pipette to each tube. The ten tubes are heated for 10 minutes at 80° C., rapidly cooled and incubated anaërobically in specimen jars with ground-glass stoppers, just large enough to take the ten tubes (Fig. 10), the oxygen being absorbed by the usual potash and pyrogallic acid mixture. The tubes are examined after two days' incubation at 37° C. for the characteristic changes described in Chapter I. In the slender test tubes advocated the 2 c.c. of milk half fills the tube, and the condition of the milk can readily be observed. The tubes being small readily go into a small specimen jar which consequently requires less chemicals to absorb all the oxygen, while space in the incubator is economised. The essential value of the modification is that the test is made much more delicate without additional work.

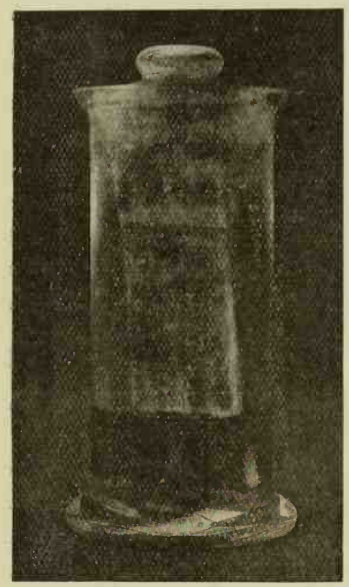

Fig. 10.

Some arbitrary standard is convenient for recording. Each positive result in a tube is counted as one *B. enteritidis* spore, an assumption which is probably, but certainly not always, true. Thus, if all the ten tubes show a positive "enteritidis change," the result is recorded as 10. All gradations between 0 and 10

[1] *Local Govt. Board Medical Officer's Report*, 1909–10, p. 477.

may be met with, and a comparative sensitive test is in this way available.

For milk work it is not usual to confirm the diagnosis by a pathogenicity test. In certain cases this may be necessary and may be carried out as described in Chapter I.

One great value of this test is that it is a non-multiplying one, so that it is especially useful for vended milk. A drawback to its utility is the fact that the spores are not always uniformly distributed in the milk.

### D. *Estimation of the Sediment or Dirt in Milk.*

Numerous investigators have attempted to judge the cleanliness of milk samples from a determination of the amount of dirt or sediment obtainable from them. The methods used are either by slow sedimentation or by centrifugalisation.

Of slow sedimentation methods, Orr's modification of Houston's method is perhaps the best. The apparatus used is shown in Fig. 11. The top of the centrifugal tube fits outside the lower part of the large glass cylinder, the two being connected by rubber tubing. The centrifuge tube is graduated in tenths and hundredths of a c.c. A litre of milk (after addition of 1 c.c. formalin) is allowed to stand for twelve hours in the large cylinder. At the end of twelve hours, a brass rod fitted with a rubber stopper at the lower end is passed through the milk, and fitting into the outlet of the cylinder prevents escape of the fluid when the centrifuge tube is detached. The tube is centrifugalised (1500 to 2000 revolutions per minute) for three to five minutes, and the milk poured off. Sodium carbonate solution (1 per cent.) is then added up to the 10 c.c. mark and the centrifugalising repeated. The deposit is then read off.

A litre of milk is a large amount to use for a single test and for routine work a rough but sufficiently reliable estimation of the volume of the sediment may be obtained by direct centrifugalisation. Tubes, which are of narrow calibre at the lower closed ends (similar in shape to those used by Orr, Fig. 11), are used, preferably with a capacity of 50 c.c. The milk is filled in to a definite volume (50 c.c.), centrifugalised for a definite period

at a known rate, and the volume of sediment directly read off in the graduated narrow end. The results are doubled and returned as volume of sediment per 100 c.c. of milk.

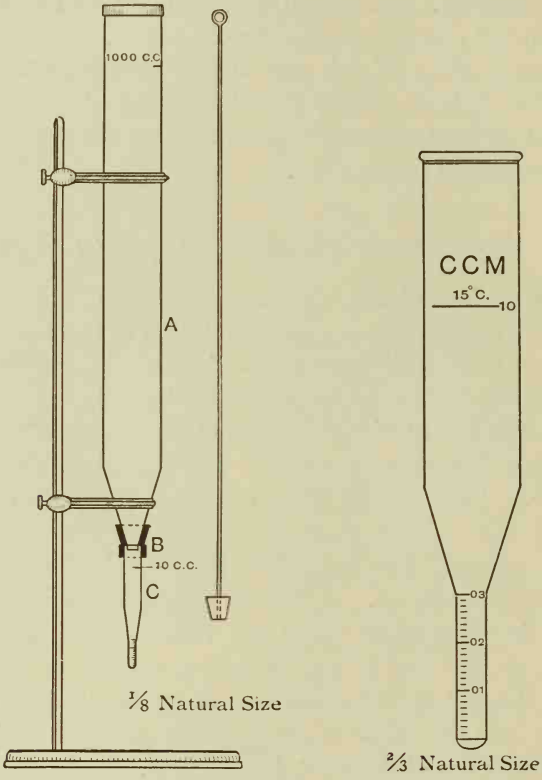


Fig. 11.

*A* = Glass tube holding a litre of milk. *C* = Tube for measuring sediment.
*B* = Rubber tubing connecting *A* and *C*.
At the side is represented a brass rod, with rubber stopper attached, for plugging the lower end of tube *A*, when *C* is taken off.

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Tube *C* is shown by the side, $\frac{2}{3}$ natural size. It is graduated in $\frac{1}{10}$ths and $\frac{1}{100}$ths of a c.c.

## E. *Determination of Acidity.*

The acidity of milk is estimated by titrating 20, 50 c.c., or other definite quantity, of the milk with $\frac{N}{10}$ sodium hydrate, phenolphthalein being used as the indicator. The titration

should be carried out in a narrow beaker with a control tube by the side. The result is conveniently expressed in terms of degrees of acidity, each c.c. of $\frac{N}{10}$ alkali required for 100 c.c. of milk being 1 degree of acidity. Thus, if 50 c.c. of milk are taken and 9 c.c. of alkali are required, then 100 c.c. would require 18 c.c., and the milk has 18 degrees of acidity. Each c.c. of $\frac{N}{10}$ alkali is equivalent to 0·009 gramme of lactic acid.

*Comparison of methods.*

The above five procedures are made use of by different workers to estimate and measure the general bacterial contamination of milk and their relative value for this purpose must be considered.

Since prejudicial milk contamination is essentially a bacteriological question it might be supposed that the estimation of the number of bacteria in milk would be a very reliable index of the degree of pollution to which it had been subjected and a measure of the undesirable condition to which it had attained. There are, however, many objections to this estimation for this purpose, the following being the most important.

(*a*) The number of bacteria enumerated varies greatly with the exact laboratory procedures adopted. Unless these are rigidly defined, widely different results will be obtained with the same milk sample. The kinds of bacteria in milk are so varied and so sensitive to slight variations of environment that quite considerable differences are readily produced.

(*b*) This estimation makes no distinction between bacterial contamination before and after the milk leaves the cow. Since, as shown above, bacteria are added from the teats and milk cistern ordinary bacterial counts cannot be taken as measuring purely external contamination.

(*c*) While under rigidly defined conditions bacterial counts are useful for samples collected at the byre this estimation is useless to gauge the extent of outside pollution for ordinary samples of market milk. The initial number of bacteria may be masked by the great multiplication which has taken place subsequently to milking. It is not in any way possible to gauge

the amount of external pollution which has been added to milk from an estimation of the number of bacteria in chance samples of vended milk.

The determination of the number of *B. coli* and allied organisms is a much more valuable estimation. These organisms as shown in Chapter I are extremely abundant in cowdung, and although there are other sources of milk contamination undoubtedly manurial pollution is by far the most prevalent. These bacilli are absent from milk samples collected directly from the teats or under conditions of great cleanliness. Further they can be easily isolated and numerically estimated by methods which are likely to give the same results with different workers.

As regards samples collected at the byre there is no doubt that this estimation is a direct and extremely valuable method of measuring the degree and extent of manurial contamination.

The value of this enumeration for chance samples of ordinary vended milk involves matters of great complexity, and it is a problem of great difficulty to correctly gauge the significance of these bacilli. If it were a practicable and universally adopted procedure to thoroughly cool milk after collection, and then to maintain it until sold at a temperature so low that *B. coli* will not multiply in it, any standards framed for *B. coli* in byre milk would be equally applicable to milk as sold. Under present conditions this is by no means the case, and it becomes a very complicated problem to say what number of *B. coli* may be allowed in vended milk samples as sold under present-day conditions.

In the writer's opinion the most reliable deductions can be obtained from a study of the rate of multiplication of *B. coli* in milk under different conditions of temperature, etc. From his investigations in this direction[1] he is of opinion that while samples at the byre should not contain more than one *B. coli* (including allied organisms) per c.c., samples of milk as vended should not contain more than 100 to 500 in winter and 1000 to 2500 in summer as extreme permissible limits.

The estimation of the number of spores of *B. enteritidis sporogenes* is also of considerable value more particularly because

[1] *Local Government Board, Medical Officer's Report* 1909-10, p. 474.

this organism does not multiply in milk and so is of equal value for byre and vended milk samples. The spores are present in considerable numbers in cowdung. If the method of enumeration described in this Chapter is adopted, 0 or 1 (*i.e.* all ten tubes negative or only 1 positive) might be considered good, 2, 3, or 4 positive as unsatisfactory, and 5 or over positive as bad.

The estimation of the sediment or dirt in milk is considered by many workers to be a very valuable guide to the bacterial contamination of milk. Since the bacteria which gain access to milk are contained in particulate matter, generally in manure, there is some justification for estimating the deposit in milk and taking it as an index of the number of undesirable bacteria which have gained access to it. The test is also a non-multiplying one, so is as valuable for vended as for byre milk samples. The amount of sediment can also be fairly easily and quickly estimated.

The objections on the other hand are considerable and important and are briefly that there is no definite relationship between the amount of sediment and the number of bacteria, that the sediment is only partially manurial, part being harmless inorganic substances or substances natural to milk, and that the amount is directly proportional to the efficiency of straining rather than to the cleanliness precautions adopted.

The last is the most important objection since straining while it removes the larger dung-particles does not remove the bacteria and makes the test of but little value.

Acidity determinations are of no real value to estimate bacterial contamination since there is no relationship between the number of bacteria or *B. coli* and the degree of acidity.

### *The detection of pathogenic bacteria in milk.*

Apart from certain types of streptococci, the only pathogenic organisms for which it is likely that milk will be required to be examined are the bacilli of tuberculosis, diphtheria, and typhoid fever.

*Examination for B. tuberculosis.* Tubercle bacilli, when present in milk, are frequently only in small numbers. It is therefore of importance to examine a considerable amount of

the milk, and to thoroughly centrifugalise it in a powerful centrifuge. The sediment is examined microscopically for the tubercle bacillus, and the rest is inoculated into guinea-pigs subcutaneously, as described below. It is advisable to inoculate several guinea-pigs from each sediment, as some of the animals may die from the action of the other bacteria present in the milk[1]. Professor Delépine has shown that this accidental mortality is much diminished if only quite fresh milk is used, or, if delay is unavoidable, when the milk samples are ice-packed during transit. This mortality can also be diminished by treating the sediment with antiformin before inoculation.

Different authorities have advocated examining different quantities of milk. The greater the amount examined the greater the chance of finding tubercle bacilli, and the more reliable a negative result. Delépine[2], as a routine method, uses two tubes of 40 c.c., and centrifugalises for a quarter of an hour in a machine giving 3000 revolutions per minute. The cream and all but 2 c.c. of the separated milk are then aspirated away, and the separate residue from each tube, containing the sediment and 2 c.c. of separated milk, after a microscopic examination of the milk has been made, is inoculated into a guinea-pig.

Other authorities use much larger quantities of milk—*e.g.* ½ litre—for each examination. Tubercle bacilli may also be contained in the cream (being carried up with the fat), and to complete the examination some of the cream may also be inoculated, or, more simply, after a preliminary centrifugalisation for half an hour, the cream may be thoroughly broken up by a sterile glass rod, and the milk again centrifugalised. In this way a large proportion of the organisms in the cream is transferred to the sediment. In any case the sediment is

[1] O'Brien (*Journ. of Meat and Milk Hygiene*, 1911, vol. I, p. 295) found that out of over 9000 guinea-pigs subcutaneously inoculated with milk sediment 33 per cent. died, from causes other than tuberculosis, before the 28th day. Careful investigation in the cause of this non-tubercular mortality showed that while part was due to infectious diseases which the animals were incubating at the time of inoculation and part to bacilli inoculated with the milk, the greater part must be ascribed to a combination of factors, including the disturbance caused to the animal's economy by the proteins injected and the consequent lowering of resistance to auto-infection.

[2] See *Transactions of the British Congress on Tuberculosis*, vol. II, pp. 286–7.

examined microscopically, and the remainder inoculated into several guinea-pigs.

To examine the sediment microscopically for tubercle bacilli, make cover-slip preparations, and either stain directly by the Ziehl-Neelsen method in the ordinary way, or, preferably, stain after a preliminary treatment with ether for several hours, to get rid of the fat.

Little or no reliance is to be placed on the microscopic examination of the sediment from mixed milk samples for the detection of the tubercle bacillus. The microscopic examination of the milk from individual cows is of more value since the chances of finding tubercle bacilli are greater, but a negative result cannot be accepted as reliable evidence that tubercle bacilli are absent.

For inoculation purposes the method of subcutaneous injection as advocated by Delépine[1] gives very reliable results. In this method the injection is made subcutaneously, with strict aseptic precautions, on the inner side of the leg at the level of the femoro-tibial articulation. If the inoculated material contains tubercle bacilli, an infection of the glands on the inoculated side takes place. The popliteal, superficial, and deep inguinal, and usually the sub-lumbar glands on the inoculated side are enlarged (see Fig. 12), and tubercle bacilli can be demonstrated in films made from them. The time at which this can be done varies with the number of tubercle bacilli injected. According to Delépine, with milk containing numerous tubercle bacilli definite evidence of tuberculosis can be seen in animals killed after ten to fifteen days; when not sufficiently numerous to be detected microscopically in the milk (*i.e.* a moderate number of bacilli) at the end of fifteen days, and when very few bacilli are present in the milk, it may be difficult to obtain any clear evidence of infection before the end of the fourth or fifth week. Delépine lays stress upon the importance of keeping the inoculated animals isolated and under favourable hygienic conditions. For ordinary work a good plan is to inoculate two animals, one being killed at the end of three weeks, and the other a week or two later.

While the pathological picture is practically conclusive of

[1] *British Medical Journal*, 1893, vol. II, p. 664.

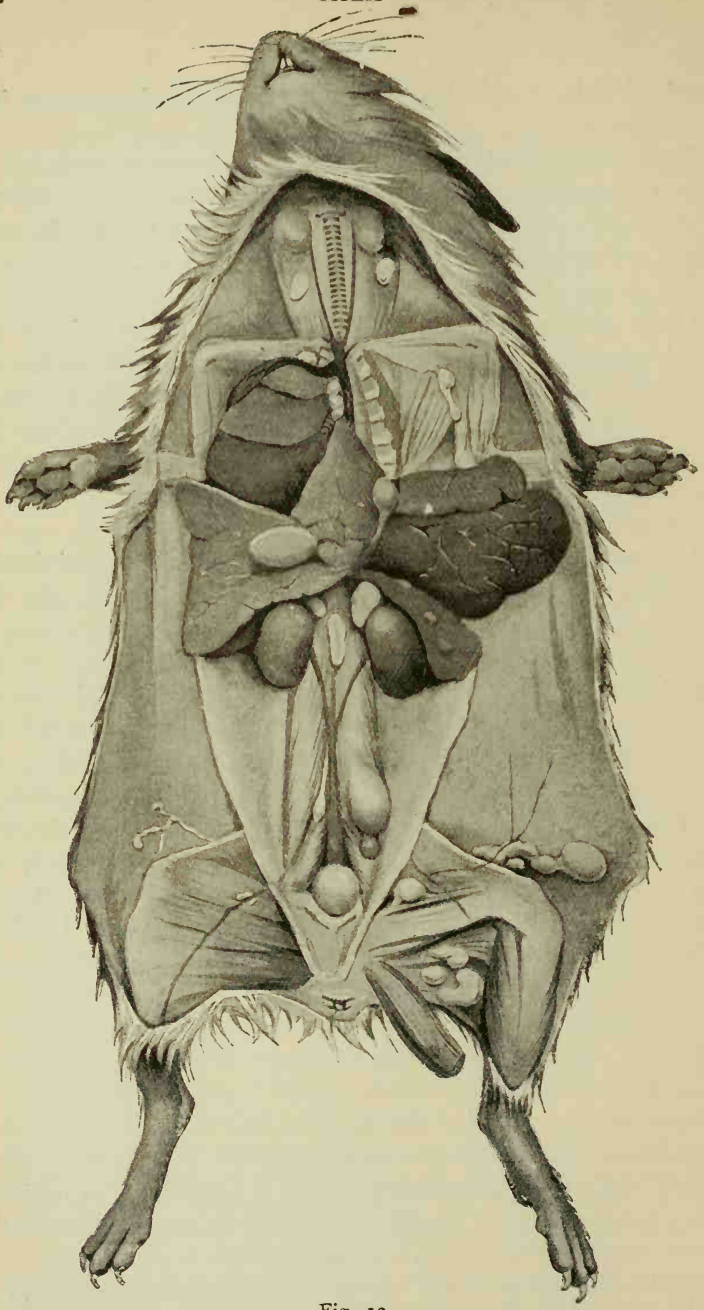

Fig. 12.

Experimental tuberculosis in a guinea-pig (about the third week after inoculation) inoculated subcutaneously in neighbourhood of the left knee-joint. (From Curtis's *Bacteriology*.)

The enlarged popliteal, superficial and deep inguinal and sub-lumbar glands on the side of inoculation are very obvious; also the retro-hepatic gland and diseased spleen.

tuberculosis, complete proof should always be obtained by finding tubercle bacilli in the enlarged glands and other tuberculous lesions.

The animal test for tuberculosis takes at least three weeks before any diagnosis can be made. To shorten this period Bloch[1] has suggested that the inguinal glands on the inoculated side should be slightly damaged by squeezing them. He found that in all positive cases the glands within 9 to 12 days were markedly enlarged and tubercle bacilli present in large numbers both in films and sections. The earlier development of tuberculosis is due to the greater growth in the slightly damaged glands. This modified procedure has been found of value by several other investigators.

The possible presence in milk of acid-fast bacilli other than the tubercle bacillus, while it diminishes the value of simple microscopic examination, does not to any considerable extent interfere with the inoculation test. The death of the guinea-pig, with lesions apparently those of tuberculosis, is almost certainly due to tubercle bacilli. As a routine procedure, and certainly in any cases of doubt, cultures should be made on glycerine agar from the enlarged glands. The simulating acid-fast bacilli grow readily and rapidly upon this and other nutrient media, unlike the tubercle bacillus.

Since the acid-fast bacilli are of considerable importance in relation to the diagnosis of tubercle bacilli in milk and milk products a brief note of this group may be of assistance.

The *acid-fast* bacilli are a group of organisms fairly widely scattered in nature and mostly of no particular significance in themselves but which owe their importance to the fact that morphologically they somewhat closely resemble the tubercle bacillus while also, like that bacillus, they resist decolorisation by acids.

Of recent years a number of such bacilli have been isolated and studied of which the best known are the butter bacillus of Rabinowitsch and Petri (see page 117), Moeller's timothy-grass bacilli I and II, originally isolated saprophytically growing upon this grass, Johne's bacillus the cause of Johne's disease (a form

[1] *Berlin. klin. Wochenschr.* 1907, Vol. XL, p. 511.

of chronic enteritis of cattle), the smegma bacilli found in smegma and the *mist bazillus* isolated from manure. In general all these bacilli morphologically resemble the tubercle bacillus but are for the most part shorter and thicker and stain more uniformly. They resist decolorisation by acids unless submitted to prolonged treatment while their resistance to decolorisation by alcohol is somewhat inferior to that of the tubercle bacillus. Injected into animals most of them, like the butter bacillus, exert pathological effects with the production of nodules resembling tubercles. The characteristic which essentially differentiates them from the tubercle bacillus is their comparatively rapid growth upon and in culture media. Their growth in days exceeds that of the tubercle bacillus in the same number of weeks. They will also grow at room temperatures. A further point of differentiation is that they do not produce tuberculin.

*Examination for B. diphtheriae.* This organism has been found in milk, but only on a very few occasions.

The method usually employed is to take advantage of the rapid growth of this bacillus on blood-serum.

The milk is centrifugalised in sterile tubes, and cultivations are made from both the cream and the sediment.

Dilution is obtained by acting upon the same principle as that used in brushing agar and gelatine plates. A little sediment or cream is taken up by a sterile platinum loop, and is rubbed in close vertical lines over the surface of three blood-serum tubes without recharging the loop. A large number of blood-serum tubes should be inoculated from both sediment and cream, incubated at 37° C., and examined after twenty to twenty-four hours. All possible *B. diphtheriae* colonies must be carefully examined (to diminish the work, surface sweepings may be taken) in cover-slip preparation. If bacilli morphologically resembling this organism are found, they must be subcultivated, obtained in pure culture, and the virulence determined.

Many observers have recorded the presence in milk of bacilli which morphologically resemble the diphtheria bacillus, but which from their other characters are certainly not that organism. Their occurrence emphasizes the absolute necessity of carrying

out cultural and animal inoculation tests before a diagnosis of diphtheria bacilli in milk is made.

*Examination for B. typhosus.* The detection of this organism in milk is a matter of considerable difficulty. The milk sediment after centrifugalisation is mixed with a little sterile water, and fractions of the emulsion are distributed over the media in a series of Petri-dishes. The media to use are one or other of those which differentiate the typhoid bacilli to some extent, such as lactose bile salt neutral red agar. All suspicious colonies are investigated. The composition of the media and the method of investigating the colonies are the same as for the isolation of this bacillus from water.

*Procedures to obtain evidence as to the condition of the milk-producing apparatus of the cows from which the milk is obtained.*

The most important of these conditions is tuberculosis of the udder. The detection of tubercle bacilli has been described, and it is only necessary to remark that finding these bacilli in milk, while generally pointing to tuberculosis of the udder of one or more of the cows supplying the milk, does not invariably bear that interpretation. Schroeder, and more recently the English Royal Commission on Tuberculosis, have shown that tubercle bacilli may be found in the milk of tuberculous cows which show no evidence of udder tuberculosis. In many cases they are derived from the fæces, which contain the bacilli and are allowed to pollute the milk.

A. *Estimation of the cellular content of milk.* All milk samples contain a certain number of cellular elements, and in certain pathological conditions their number is enormously increased. Deductions of great value can be made from accurate determinations of their number in the milk of individual cows. For mixed milk samples this determination is of much less utility.

The older method used was to centrifugalise the milk, spread the sediment as evenly as possible over a cover-slip, dry, and stain by methylene blue. The number of leucocytes in a number of fields of vision was then counted, and an attempt made to

estimate by returning the number of leucocytes as so many per microscopic field. This method is very inaccurate, since the number will vary with the size of the microscopic field, and especially with the thickness of the film, the latter being impossible to accurately control. The writer worked out the following method, by which the cellular elements can be readily and accurately enumerated in milk.

*Savage's Method*[1]. The ordinary Thoma-Zeiss blood-counting chamber is employed. Direct counting of the cells is impossible owing to the opacity caused by the large amount of fat. One c.c. of the milk is accurately transferred to a centrifugal tube (about 15 c.c. capacity) of the pattern shown in Fig. 13, and freshly filtered Toisson's solution[2] is poured in to almost fill the tube. The two fluids are well mixed and then centrifugalised for 10 minutes. The cream is well broken up by a clean glass rod, to disentangle leucocytes carried to the surface, and the mixture centrifugalised for an additional 5 minutes. All the fluid is then removed down to the 1 c.c. mark, great care being taken not to disturb the deposit. This can be conveniently and readily done by means of a fine glass tube connected to an exhaust pump. Theoretically, all the cellular elements present in the original 1 c.c. of milk are now present in the 1 c.c. of fluid. The deposit is thoroughly well mixed (with a wire), and distributed through the 1 c.c. A sufficient quantity is placed on the ruled squares of the Thoma-Zeiss apparatus, and the cover-glass put on. The number of cells is counted in a number of different fields of vision, moving regularly from one field of vision to another. The diameter of the field of vision is ascertained before counting by drawing out the microscope tube

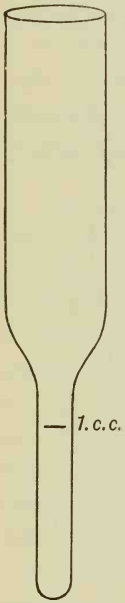

Fig. 13.

[1] *Journal of Hygiene*, 1906, Vol. VI, p. 123.

[2] This is the well-known indifferent solution used in blood enumerations. It does not injure the cells, but stains them sufficiently to render them clearly visible. Its composition is methyl violet 0·025 grm., sodium chloride 1 grm., sodium sulphate 8 grms., glycerine 30 c.c., distilled water 160 c.c.

until an exact number of sides of the squares spans a diameter of the field of vision.

The number of cellular elements per cubic mm. of milk $= \frac{56{,}000y}{11d^2}$, where $y$ = the average number per field of vision, $d$ = the number of squares which just spans the diameter. $d$ is determined once for all by marking the microscope draw tube so that only 20 fields have to be counted, and the figures substituted in the formula.

B. *Examination of the stained centrifugalised deposit.* To obtain comparable results the sediment from a definite amount of milk should be examined after centrifugalisation for a definite period. Ten c.c. of milk centrifugalised for 10 minutes is convenient. Part of the deposit is spread thinly but uniformly over a cover-slip, dried in air, fixed in the flame, or preferably by soaking in a mixture of equal parts alcohol and ether for one minute, stained by methylene blue and mounted in balsam.

The preparation may be utilised to gain an idea of the general bacterial content, whether streptococci are present, and if so in what numbers and whether intracellular, while, if considered necessary, a differential count may be made of the cellular elements present. For this purpose not less than 200 should be enumerated. With care a rough but valuable estimate can be obtained from this examination as to the probable number of bacteria in the sample.

C. *Determination of the number of streptococci.* To estimate the number of streptococci in milk the method recommended as the simplest and most reliable is to add diluted fractions of the milk, 1·0, 0·1, 0·01, 0·001 c.c. etc., to tubes of glucose neutral red broth. Ordinary broth will do, but the neutral red broth is preferable and gives better results. The tubes are incubated for two days at 37° C. and then examined, in hanging drop preparation, for streptococcus chains. The deposit should be selected for examination, and several hanging drop preparations made. A positive result should only be recorded when quite definite chains of cocci are detected, or, in doubtful cases, when stained preparations show such definite chains.

To isolate the streptococci, brush diluted loopfuls of the positive tubes over plates containing nutrient agar. Incubate for 24 hours, and if necessary for 2 days, at 37° C. Subcultivate the colonies with the characters of streptococcus colonies into broth or upon sloped agar in tubes containing condensation water. In cases in which streptococci are likely to be scanty, part of the centrifugalised deposit may be used to inoculate the agar plates.

The tests recommended to differentiate the streptococcus strains isolated are the following: morphology, growth upon sloped nutrient agar, growth in nutrient broth, growth upon gelatine slope, action upon litmus milk, the production of acid in lactose, saccharose, salicin, mannite, raffinose, and inulin.

The sugar-alcohol media for the differentiation of streptococci were introduced by Gordon. Their method of preparation is given in the Appendix.

For some purposes it is of great value to ascertain the pathogenicity of isolated strains of streptococci. This is conveniently done by injecting mice subcutaneously or intraperitoneally.

The presence or absence of streptococci in milk may also be studied by a careful examination of the centrifugalised deposit stained by methylene blue. Failure to find streptococcus chains does not mean they are absent, but only suggests they are not present in considerable numbers. The stained deposits from samples of vended milk, usually show numerous streptococci, but in those made from fresh byre milk samples they are as a rule not to be demonstrated.

*Comparative value of above methods.*

The estimation of the cellular content is of great value in the examination of the milk of individual cows. Cellular elements are invariably present in milk even from perfectly healthy cows but as a rule not in large numbers. Numbers varying from 50 to over 1000 per cubic mm. have been recorded by the writer from the milk of individual healthy cows. Any considerable increase is due to physiological or pathological changes (old or recent) in connection with the udder or teats.

Examples of physiological conditions are advanced pregnancy or the first few days after calving. Pathological causes are any

local inflammatory or suppurative conditions. For these conditions the number of cells present may be very high and in cases of definite mastitis numbers as high as 300,000 per cubic mm. have been found by the writer.

This estimation may be very useful to detect the commencing stages of mastitis and it may be said that in general when cellular elements are present in numbers more than 800 or so per cubic mm. they indicate the need for careful inquiry as to the local conditions of the cow's udder or teats.

For ordinary mixed milk samples this determination is of no particular value.

The significance of streptococci in milk is a complex and difficult subject. They are undoubtedly very prevalent not only in ordinary samples of market milk but also in samples of mixed milk collected immediately after milking when no multiplication in the milk can have taken place. For example the writer found streptococci present in every sample of mixed milk (although many were collected at the farm) in a large series which he examined, when 1 c.c. was sampled and in over 80 per cent. when 0·1 c.c. was examined. Also streptococci were present in over half the samples of milk examined drawn direct from the teats of cows.

It would appear that there are four main sources of streptococci in milk, i.e. the teat passages and milk cisterns of healthy cows, manurial contamination (streptococci are present in cow manure 0·1 to 10 millions or more per gramme), stale milk from unclean milk vessels and local disease of the cow (mastitis, etc.).

It is obvious from these varied sources of origin that the presence of streptococci in moderate numbers in milk samples cannot be accepted as of necessity of prejudicial significance or as pointing, without further investigation, to local disease in one of the cows supplying the milk.

Whether their presence *in very large numbers* in fresh milk can be taken to indicate inflammatory disease of the milk-secreting organs of one or more of the cows supplying is a question which cannot be said to be at present settled, but it is certainly an unsatisfactory condition and should lead to careful examination of the cows.

In cow mastitis and some cases of teat ulceration the lesions show streptococci in enormous numbers. In one instance the writer found that when the cows of a cow-keeper whose fresh milk contained enormous numbers of streptococci were examined severe teat ulceration, associated with abundant streptococci, was found upon several of the cows.

The differentiation of the streptococci found in mixed milk samples is scarcely sufficiently advanced to furnish reliable guidance as to which varieties are prejudicial but such differentiation is more valuable when dealing with samples from individual cows.

Mastitis, or garget, in cows may be due to a number of different organisms, but in the writer's investigations 75 per cent. of the cases were due to streptococci, while 80 per cent. of the streptococci formed one common type, the *Streptococcus mastitidis*. This is a long chain streptococcus, growing rapidly in broth, forming a coherent deposit, but leaving the upper part clear. It grows upon gelatine without liquefaction, produces acid in milk, and clots it within three days, gives no neutral red reaction, and produces acid in lactose and saccharose media, never in mannite, and not usually in salicin, raffinose, or inulin. It is non-pathogenic to mice. Inoculated into the teats of goats, it sets up mastitis on the infected side[1].

In investigating human outbreaks of sore throat, etc., spread by milk and associated with the presence of a cow with mastitis in the herd supplying the milk the best way to establish or confute any causal relationship is to *promptly* ascertain if the streptococci from the human disease can cause mastitis in goats by infection of the teats. If they are of milk origin they should produce, if not mastitis, at least a marked inflammatory reaction in the inoculated goat[2].

*Summary of procedures recommended.*

A number of different methods have been discussed. For the convenience of those not versed in the examination of milk samples the following procedures are recommended:

[1] For detailed consideration of the bacteriology of mastitis see *Local Govt. Board Medical Officer's Report* 1906–7, 1907–8.

[2] *Local Govt. Board Medical Officer's Report* 1908–9.

I. *Ordinary mixed milk samples for general bacterial contamination.*

Make dilutions A, B, C, D as above.

(*a*) Examine the stained centrifugalised deposit from 10 c.c. If streptococci are very numerous enumerate them using a series of neutral red broth preparations.

(*b*) Estimate the number of *B. coli* and allied organisms.

(*c*) „ „ *B. enteritidis sporogenes* spores.

II. *Samples to be specially examined for the tubercle bacillus.*

Examine both microscopically and by animal inoculation.

III. *Special examination of the milk of individual cows.*

(*a*) Carefully examine the stained centrifugalised deposit, including a differential cellular count.

(*b*) Estimate the number of *B. coli* and allied organisms.

(*c*) Estimate the cellular content.

(*d*) Examine for and estimate the number of streptococci. Brush definite fractions over plates of suitable solid media and isolate the chief varieties present.

(*e*) If necessary examine specially for *B. tuberculosis.*

## CHAPTER VII

### MODIFIED MILK AND MILK PRODUCTS

#### I. Condensed Milk.

Condensed milk is prepared by concentrating either whole or separated milk, with or without the addition of sugar. Theoretically, therefore, there are four kinds of condensed milk, but the writer is not aware of any unsweetened separated brands. The four kinds are:

(*a*) Sweetened condensed whole milk.

(*b*) Unsweetened condensed whole milk.

(*c*) Sweetened condensed separated milk.

(*d*) Unsweetened condensed separated milk.

Bacteria are present in all varieties of condensed milk, but in relatively small numbers, the method of preparation being sufficient to eliminate most of the bacteria present in the original milk. The following figures have been recorded:— 230 to 70,940 per c.c. by Heggs, 130 to 20,000 per gramme by Klein, 20,000 to 120,000 per c.c. by Dold and Garratt. Dold and Garratt[1] found *B. coli* and *B. enteritidis sporogenes* absent from 1 c.c. in all the 19 samples examined, but streptococci were found in 1 c.c. in 8 cases (32 per cent.) and in 0·1 c.c. in 3 cases. Pathogenic organisms could not be detected in any of the samples, either by direct examination or animal inoculation.

Gordon and Elmslie[2] examined 15 different brands of condensed milk, and found none of them sterile. Streptococci with characters similar to those found in milk were present in all the 11 sweetened samples. These investigators concluded that during the process of condensing unsweetened milk, sterility is secured, the organisms found being subsequently introduced from the air, but that in the condensing of sweetened milk sterility is not attained, some at least of the organisms in the original milk surviving the process of condensation. No organisms of the *B. coli* group were isolated from any of their samples.

Delépine[2] carefully investigated the resistance of tubercle bacilli to the processes involved in the preparation of condensed milk, and found that although the original milk had been made artificially highly virulent, after condensation its power to set up tuberculosis in guinea-pigs was lost, all the tubercle bacilli being killed.

The analysis of condensed milk is mainly chemical, bacteriological examinations being seldom required.

The bacteriological examination of condensed milk should be on the same lines as for ordinary milk, the sample being first thoroughly mixed with a definite quantity of sterile water.

[1] *Journ. Roy. Inst. Public Health*, 1910, XVIII, p. 294.

[2] *Report* by Dr Coutts to Local Govt. Board on Condensed Milk, 1911.

Care must be taken to thoroughly cleanse and sterilize the outside of the tin before opening, while the instrument to open the tin, which may conveniently be an ordinary tin-opener, must be sterilized before use.

The fact that condensed milk is a culture medium for bacteria—although not a very suitable one—is of much greater importance than the fact that it contains a few bacteria. The latter fact is evidently of no public health significance.

## II. Dried Milk.

Dried milk is now manufactured commercially to a considerable extent. It is the powder obtained either by passing milk rapidly between heated surfaces so that it is deprived of its water, or by drying on a cylinder in a partial vacuum. This dry powder, on being again mixed with water, is converted into a fluid which looks like milk, and which, on ordinary chemical analysis, shows the chemical constituents of that substance. In other respects considerable alterations have taken place, for example the enzymes have been destroyed, the fat globules physically altered, etc.

To examine bacteriologically the dried milk would have to be dissolved in sterile water, and the mixture examined by methods similar to those employed for milk.

The chief examinations which might be required would be to determine if sterile, or if not the number and kinds of bacteria present, and also to ascertain if tubercle bacilli were present.

From the limited number of experiments which have been made it appears that tubercle bacilli are killed in the process of manufacture.

## III. Cream.

The bacteriological examination of cream is of considerable importance, since it is usually richer in bacteria than either whole milk or separated milk. This is shown by direct bacterial examinations, while in guinea-pig inoculation experiments it is not uncommon to find that a considerably higher percentage

succumb to acute infections when inoculated with cream than when inoculated with the sediment of whole milk. In examinations of milk samples for pathogenic bacteria, such as *B. diphtheriae*, *B. tuberculosis*, etc., the examination of the cream should never be neglected, while it follows that market-cream itself should be more often examined than is the case.

Anderson[1] gives the following figures as to the number of bacteria in cream. In 26 samples of milk the average number of bacteria in the sediment and in the cream layer obtained from gravity and centrifugally raised cream was—

| | | | |
|---|---|---|---|
| Gravity raised | cream layer ... ... ... | 68,690,000 | bacteria. |
| | sediment layer ... ... | 4,840,000 | ,, |
| Centrifugalised | cream layer ... ... ... | 96,840,000 | ,, |
| | sediment layer ... ... | 18,840,000 | ,, |
| Whole milk | ... ... ... ... ... | 14,388,000 | ,, |

In Washington, U.S.A.[2], the examination of 130 samples of cream between February and July, 1907, showed that while all showed over 10,000 organisms per c.c. only three contained less than 50,000, while 10 per cent. contained between 10 and 25 million bacteria per c.c. The average for all the samples was 12,130,000 bacteria per c.c.

The bacterial content of cream varies enormously with its age. Quite fresh centrifugalised cream usually has a bacterial content lower than that of the milk from which it is obtained. According to Swithinbank and Newman the result of centrifugalising milk is that roughly 60 per cent. of the organisms will be found in the sediment, 25 per cent. in the separated milk, and 15 per cent. in the separated cream. Subsequently, however, on keeping the bacteria multiply enormously, cream being a good nutrient medium. The Commission on Milk Standards appointed by the New York Milk Committee recommended as a standard for cream that it should not contain more than 300,000 bacteria per c.c.

Cream can be bacteriologically examined by methods similar to those used for milk, the cream being diluted with sterile water. For examination for tubercle bacilli the cream itself

[1] *Bulletin 56 Washington Treasury Department*, 1909, p. 739.
[2] *Ibid.* p. 259.

may be directly injected into guinea-pigs. A chemical examination for preservatives should always be carried out before the bacteriological examinations are undertaken.

## IV. Butter.

The bacteriology of butter comes under consideration from two points of view. Part of the changes which occur in the conversion of the cream into butter are bacterial in origin, and much investigation has taken place as to the precise bacteria involved, the changes they cause and the influence of the other kinds of bacteria present. This economic bacteriology of butter is of the utmost importance in relation to the production of good butter, but can only be briefly mentioned here. The other point of view is concerned with the part butter may play as a vehicle for the transmission of pathogenic bacteria.

As pointed out in the preceding sub-section, bacteria are very numerous in cream, and are of many different varieties. Some of these are active participators in butter ripening, others are prejudicial and may cause abnormal flavours, etc. Butter can be made from either sweet or soured cream. Sweet cream butter is prepared at once, while to make the latter the cream is allowed to become sour, by the development and chemical activities of the lactic acid group of bacilli, before it is converted into butter.

If the cream is pasteurised first and the requisite bacteria subsequently added in the form of the so-called "starters," the process is better controlled, and therefore more uniform quality butter is produced, abnormalities of taste, odour, etc., due to foreign bacteria are prevented, while the keeping qualities of the butter are improved.

In America to some extent, and in Denmark for the most part, the butter is made from pasteurised cream to which starters are added, while in England it would appear that the greater part of the butter is made naturally, that is by trusting to the suitable bacteria being naturally present.

The "starters" are more or less pure cultures of lactic acid bacteria.

English butter usually contains when fresh a very large number of bacteria, often one to ten million, and sometimes as many as 40 million or more per gramme. The majority of these are lactic acid bacilli such as *B. acidi lactici*, *B. lactis aerogenes*, etc. The number of bacteria materially decrease with keeping.

Butter "faults" are not infrequently met with. The majority of these are due to faulty management, resulting in the development of unwelcome species of bacteria which produce rancidity, abnormal flavours, bitter taste, etc. Of these unfavourable bacteria may be mentioned *B. subtilis*, *B. mesentericus*, *B. fluorescens liquefaciens*, etc., and probably some yeasts. Some of these changes, for example rancidity, are due to the inter-action of several kinds of bacteria. These unfavourable bacteria are usually kept in check by the lactic acid bacteria.

If the cream is pasteurised and pure cultures added there is obviously much less risk of these abnormal bacteria developing and producing butter faults.

No data of special value are likely to be obtained from an enumeration of the bacteria in ordinary butter samples. From the public health point of view the bacteriological examination of butter is at present limited to its examination for pathogenic bacteria, particularly *B. tuberculosis*.

### *Pathogenic bacteria in butter.*

*B. tuberculosis.* The investigations of numerous workers have proved that tubercle bacilli are not infrequently present in butter. Different investigators have, however, obtained very varying results, their percentages of positive results varying from about 8 to 30. There are few available English records, but it is probably safe to state that at least 10 per cent. of samples contain living virulent tubercle bacilli. A recent report of the Local Government Board[1] states that a number of butter samples were examined in the Board's Laboratory. Of 48 samples of foreign butter four were "inconclusive," the others showed no tubercle bacilli. Of 60 samples of British origin 22 were "inconclusive," while two showed the presence of tubercle bacilli.

[1] *Local Govt. Board Medical Officer's Report*, 1911–12, p. 184.

Experiments have shown that the tubercle bacillus will remain alive for considerable periods in butter, i.e. five months or longer (Mohler, Washburn and Rogers). Teichert on the other hand only found the bacilli alive up to 18 days.

To detect tubercle bacilli in butter the inoculation method is the only satisfactory one. The butter is placed in centrifugal tubes which are stood in warm water at 42° C. until the butter is completely melted. The material is centrifugalised when liquid, and the sediment inoculated into guinea-pigs as described under milk. It is difficult to keep the butter liquid during the centrifugalisation.

It is important to remember that acid-fast bacilli may be present in butter, and that not only may they interfere with a simple microscopic examination, but that they may produce pathogenic lesions closely resembling those caused by the tubercle bacillus including the death of the animal. The *Butter bacillus* isolated independently by Rabinowitsch and Petri from butter is an acid-fast organism which morphologically resembles the tubercle bacillus, and which will produce similar lesions in guinea-pigs when injected intraperitoneally mixed with butter. It can be readily distinguished from the tubercle bacillus by the comparative rapidity of its growth on ordinary media, such as nutrient-agar or glycerine-agar, a well-marked thick crinkled growth being present after three to four days.

In every case in which butter is being examined for *B. tuberculosis* it is very important not only to make microscopic films from the affected organs and demonstrate acid-fast bacteria, but also to make cultivations upon glycerine-agar, to ascertain if the butter bacillus is present and the cause of the lesions. It is also advisable to subcultivate upon blood serum and egg medium to isolate any tubercle bacilli present.

*Other pathogenic bacteria.* The diphtheria bacillus appears to have but a short life in butter, but the typhoid bacillus has been shown by different observers to live for at least ten days. No definite outbreaks appear to have been traced from their presence in butter.

To examine, liquefy at 42° C. and centrifugalise as for tubercle

bacilli in butter. The sediment and liquid are pipetted off from the fat and re-centrifugalised. The sediment is then examined by methods similar to those described under water and milk.

## V. Cheese.

The changes which occur in cheese ripening appear to be partly chemical, partly bacteriological, the latter being more particularly concerned with the processes which give the different flavours. There appears to be great obscurity and difference of opinion as to the bacteria concerned and the actual parts they play.

The number of bacteria in cheese is always large, but varies with the age of the cheese. According to Russell[1] the ripening process can be divided into a period of initial bacterial decline soon followed by a period of great bacterial increase, and ending with a period of bacterial decline.

A large number of different bacteria has been described in cheese, the lactic acid bacteria forming the largest group. In addition gas-producing bacteria, bacteria decomposing casein, moulds, etc., are all numerous.

From the public health point of view the bacteriological examination of cheese is seldom undertaken, and is of little value. The only examination of any importance is for *B. tuberculosis*. Hormann and Morgenroth found tubercle bacilli in 3 out of 15 samples, Rabinowitsch in 3 out of 5 samples, and Eber in 2 out of 50 samples.

Tubercle bacilli may also live for some time in cheese. Using milk artificially inoculated with tubercle bacilli and then made into cheese, Galtier found the bacilli alive in cheese 2 months and 10 days old. Harrison made cheese from milk artificially inoculated with tubercle bacilli. In cheese made by the Emmentaler method, they died between the 34th and the 40th day; in Cheddar cheese, after 62 to 70 days.

Mohler, Washburn and Doane[2] have shown that tubercle bacilli may live for even longer periods. In their experiments

[1] *Agric. Exp. Station, Wisconsin, 13th Annual Report*, 1896, p. 105.

[2] *Annual Report, Bureau of Animal Industry, U.S.A.*, 1909.

they found that infected cheese 220 days old set up, when inoculated, marked generalized tuberculosis, and when 261 days old slight tubercular lesions.

To examine cheese for tubercle bacilli the method of Mohler, Washburn and Doane may be used. Portions of the central part of the cheese are rubbed up in a mortar with sterile normal saline solution. After being well ground the liquid is strained through a layer of absorbent cotton and the equivalent of 2 grm. of cheese injected into each guinea-pig.

## VI. Ice-cream.

The term ice-cream covers articles which are to some extent prepared differently and which contain different constituents, but these differences of composition are not extensive as regards ice-creams made in this country.

In its simplest form ice-cream consists of milk and sugar, with a thickening agent such as corn-flour and with or without eggs. Flavouring agents and sometimes colouring matters are added, while gelatine is sometimes used in the manufacture.

Cream, as such and apart from milk, does not appear to be used in the preparation of the article vended in this country as "ice-cream." While the mixture is always heated the duration of the heating varies greatly, and frequently some constituents are added afterwards. The mixture is cooled and subsequently frozen.

From the bacteriological point of view the chief points of importance are that the constituents form a food highly nutrient for bacteria, that it is often made from materials already heavily infected with bacteria, that this food is heated but not sterilized, that the cooling is natural and therefore prolonged, and that the subsequent freezing has no inhibitory action upon any of the harmful bacteria which it may contain.

### *Bacterial content.*

Buchan found that the average period of cooling before freezing was about $20\frac{3}{4}$ hours. From numerous reports by Medical Officers of Health it is evident that both the preparation

of this food and the prolonged period of cooling take place in very many cases under most insanitary conditions. It is not therefore to be wondered at that ice-cream is frequently found to be very heavily contaminated with bacteria, and that a number of outbreaks of illness has been reported from its use.

The following data from a valuable paper by Buchan[1] will give some idea of the average bacterial condition of ice-cream:

| | *a* | *b* | *c* |
|---|---|---|---|
| Organisms per c.c. (averages) ... | 867,000 | 13 millions | 372 millions |
| Percentage showing:— | | Percentages | |
| Coli group organisms in 1 c.c. or less | 16 | 58 | 94 |
| "Enteritidis change" 10 c.c. or less | 46 | 64 | 80 |
| ,, ,, 100 c.c. ... | 82 | 92 | 96 |
| Streptococci in 0·01 c.c. or less ... | 6 | 36 | 68 |
| ,, ,, 1 ,, ... | 38 | 52 | 82 |

*a* = fifty samples collected immediately after heating.
*b* = ,, ,, ,, at varying intervals after cooling had begun.
*c* = ,, ,, ,, after the material had been frozen.

Pennington and Walter[2] examined sixty samples of commercial ice-cream in America, finding the number of bacteria to vary from 50,000 to 151,000,000. Streptococci were found in 80 per cent. of the samples.

### *Bacteriological examination.*

The ice-cream should be collected in a sterile vessel—*e.g.*, a wide-mouthed sterile bottle with glass stopper—and packed in ice if it cannot be examined at once.

To examine, melt the ice-cream by placing for fifteen to twenty minutes in the 22° C. incubator, then treat as a milk sample.

The degree of dilution and the methods of examination are similar to those used in the examination of milk.

The examinations usually carried out are:—estimation of the number of bacteria, estimation of *B. coli* group organisms, with identification if necessary of the chief kinds present, enumeration of streptococci, estimation of spores of *B. enteritidis*

[1] *Journ. of Hygiene*, 1910, X, p. 93.
[2] *New York Med. Journ.* 1907, LXXXVI, p. 1013.

*sporogenes.* All these are carried out as for milk samples. Pathogenic bacteria such as *B. tuberculosis*, *B. diphtheriae* and *B. typhosus* occasionally have to be examined for, using the same procedures as described under milk.

In addition *B. enteritidis* and other Gaertner group bacilli will occasionally have to be examined for. A number of food poisoning outbreaks have been ascribed to the consumption of ice-cream, and some if not most of these are due to infection of this food with Gaertner's group bacilli. The method of examination to employ is given in Chapter VIII.

*Bacterial standards.*

It is not at present practicable or expedient to lay down definite standards of number of bacteria or of special kinds to be permitted in ice-cream. Rough standards as a guide to the sanitary conditions and cleanliness precautions observed would however be of great utility. For these purposes standards based upon the number of *B. coli* group organisms would probably be of greatest service.

Buchan has suggested that ice-creams made under clean conditions:—

(*a*) Should not contain more than 1,000,000 organisms per c.c. capable of growing on nutrient gelatine (reaction + 1 per cent.) at 20° to 22° C. in three days.

(*b*) Should not contain more than 1,000,000 organisms per c.c. capable of growing on nutrient agar (reaction + 1 per cent.) at 35° to 37° C. in two days.

(*c*) Should not produce acid and gas in bile-salt glucose broth with a less quantity than 0·1 c.c.

(*d*) Should not contain the *Bacillus enteritidis sporogenes* in less than 10 c.c.

(*e*) Should not contain streptococci in less than 0·001 c.c. of ice-cream.

# CHAPTER VIII

## THE BACTERIOLOGY OF MEAT AND MEAT PRODUCTS

Until the last few years but little was known of the normal bacteriology of meat and meat products and much work is still required before many important points related to meat conditions can be regarded as settled. The text books contain little or no reliable information and much of the following is contained in reports and journals not readily accessible. For this reason rather more extended information is supplied than perhaps would otherwise be warranted by the importance of the subject-matter.

### I. *The bacteriology of meat, fish, made-meats, etc., derived from healthy animals.*

Little or no work in this direction has been done in this country, but under the better regulated meat inspection arrangements which prevail in Germany numerous investigations have been carried out in recent years. The following will illustrate the findings.

#### *Unprepared meat.*

Gaertner in 1908 found that bacilli were present on the surface of fresh meat but had not penetrated, if the examination was made within three days of slaughter, while by the end of ten days they had not penetrated more than 0·5 inch into the interior. These results were confirmed by Forster (1908) as regards the general sterility of the interior of fresh meat.

Conradi[1] in 1909 used a special enrichment method (see page 128) to detect bacilli and obtained different results. He examined 162 organs from 150 healthy animals (bovines, calves and pigs) slaughtered at Neunkirchen Slaughter House and

[1] *Zeit. f. Fleisch. u. Milchhyg.* 1909, July, p. 341.

found bacteria in 72 cases. Both aërobic (42 strains) and anaërobic bacilli (30 strains) were isolated. The aërobic bacilli were *B. coli communis*, *B. lactis aerogenes*, *Streptococcus acidi lactici*, *B. mesentericus*, *B. fluorescens non-liquefaciens*, *Diplococcus pneumoniae* and *B. suipestifer*. The anaërobic bacilli were chiefly of the butyric acid group but the varieties were not accurately defined.

Bierotti and Machida[1] employed a modified Conradi method and obtained very similar results. They examined 54 organs from 11 quite healthy animals and found bacilli in 32.

Their results and those of Conradi are grouped together in the following table.

| Organ | No. of specimens examined | Bacteria found | Percentage positive |
|---|---|---|---|
| Liver ... ... ... | 74 | 50 | 67·5 |
| Muscular tissue ... | 70 | 25 | 36 |
| Kidneys ... ... | 28 | 10 | 36 |
| Lungs ... ... ... | 16 | 12 | 75 |
| Lymphatic glands ... | 4 | 1 | 25 |
| Spleen ... ... ... | 22 | 6 | 27 |
| Testicles ... ... | 1 | 0 | 0 |
| Heart ... ... ... | 1 | 0 | 0 |
| | 216 | 104 | 48 |

Horn[2] examined oxen slaughtered in the Leipzig Slaughter House, using a modified Conradi method (enrichment method) and also direct examination. He found, as might be expected, that the number of bacteria in the muscles varied with the time since slaughter. His results are briefly summarised in the following table.

*Musculature of healthy animals.*

| Time after slaughter when samples removed | Number examined | | Positive results | | Percentage positive | |
|---|---|---|---|---|---|---|
| | with enrichment | without enrichment | with enrichment | without enrichment | with enrichment | without enrichment |
| 24 hours | 36 | 36 | 5 | 2 | 14 | 5·6 |
| 1—3 days | 7 | 7 | 0 | 0 | 0 | 0 |
| 3—6 ,, | 13 | 13 | 4 | 2 | 30·7 | 15·4 |
| 6—21 ,, | 24 | 24 | 13 | 6 | 54 | 25 |

[1] *Muench. Med. Woch.* 1910, vol. LVII, p. 636.

[2] *Zeit. f. Infekt. Krank. der Haustiere*, 1910, vol. VIII, p. 424.

No proper differentiation of the isolated bacteria was carried out.

These results may be compared with those obtained by the same observer in regard to emergency slaughtered animals, *i.e.* animals killed on account of some diseased condition. Examining 49 specimens of musculature Horn obtained 31 per cent. of positive results (31 with and 21 without enrichment methods).

Zwick and Weichel[1] also investigated this matter using five different methods. Their results agree with those of Conradi as regards the frequency of the occurrence of bacteria in the liver, but not as regards the other organs. For example, in only one out of the 63 musculature specimens examined were bacilli found (*B. coli* group).

The general results obtained by different investigators show that bacteria may be present on the surface of meat, this being largely a question of cleanliness and amount of contact with bacteria-containing materials, and also in the depth of the meat. As regards musculature the evidence is not concordant, but as regards the liver, and to a lesser extent other organs, it is evident that they may contain bacilli, some of which are possibly pathogenic to man. The chief value of the above observations is however rather to show that in examining organs from possibly diseased animals too much stress must not be attached to the mere presence of bacilli without corroborative evidence as to their disease-producing *rôle*.

As regards fish Ulrich[2] found that the number of bacteria in or on raw fish is considerable even at ordinary temperatures; the bacilli present being largely *B. coli* and *B. proteus* group organisms.

### *Prepared meat.*

**Chopped meat** (Hackfleisch). German bacteriological examinations do not usually mention the actual numbers and kinds of bacteria present, but it is evident that very large numbers of bacteria will be present. Gaertner group bacilli

[1] *Arbeit. a. d. Kais. Gesund.*, 1911, vol. XXXVIII, p. 327.
[2] *Zeit. f. Hyg.* 1906, LIII, p. 176.

have been found by some workers but not by others when adequate differentiating tests are used. Zweifel[1] at Leipzig examined 248 specimens of "hackfleisch" obtained quite fresh, and in 165 cases found *B. proteus* and other bacilli, all non-pathogenic to mice by feeding.

**Sausages.** According to the writer's own examinations, bacilli are present in large numbers and the kinds present usually include bacilli of excretal type. The bacterial content in *B. coli* group organisms in 27 sausage samples obtained from different sources examined by the writer[2] was as follows:—

| *B. coli* organisms | | | | | No. of specimens |
|---|---|---|---|---|---|
| Less than | 10 | per grm. | of sausage-meat | | 0 |
| ,, | 10—100 | ,, | ,, | ,, | 4 |
| ,, | 100—1000 | ,, | ,, | ,, | 6 |
| ,, | 1000—10,000 | ,, | ,, | ,, | 4 |
| ,, | 10,000—100,000 | ,, | ,, | ,, | 7 |
| Over | 100,000 | ,, | ,, | ,, | 2 |
| Over 100 but number not estimated | | | ... | ... | 4 |

The number of organisms present was determined for a few of the samples and varied from 360,000 to over 600,000 per gramme.

Streptococci were also present in large numbers in a majority of the samples examined.

These results were obtained with sausages examined quite freshly prepared, all being made the same day.

Evidently in sausages as usually manufactured there is a considerable access of material containing excretal bacilli.

**Brawn.** Brawn is or should be thoroughly boiled when prepared so that whatever its original bacterial contamination it should be free from *B. coli* and non-sporing bacteria when made. As a rule this is the case, but brawn is a material very favourable to bacterial multiplication so that if, after preparation, it is placed in positions liable to bacterial contamination specimens will soon show a high bacterial content. Of 11 samples examined by the writer *B. coli* was absent in 0·1 grm. in seven,

[1] *Centralb. f. Bakt.* 1911, vol. LVIII, p. 115.

[2] *Journ. Roy. San. Inst.* 1908, vol. XXIX, p. 366, and *Local Govt. Board Medical Officer's Report*, 1909–10, p. 446.

present 1—10 per grm. in one, present 100—500 in one, present 5000—10,000 in one, while in the remaining sample these organisms were about 50,000 per grm. In several instances the brawn samples were taken from open shelves, etc., in places obviously markedly exposed to dust and possibly other forms of contamination.

In regard to other made foods very little data seem to be available as to their bacterial content under ordinary conditions.

In ordinary brines used for salting meat *B. coli* group organisms are frequently present in large numbers.

II. *The bacteriological examination of meat and meat products.*

Such an examination may be required for the following purposes:

(A) Examination for pathogenic bacteria.

(B) To study the general bacterial content, degree of bacterial contamination, etc.

### A. *Examination for pathogenic bacteria.*

The examination of meat for pathogenic bacteria can obviously only be profitably undertaken in cases in which the animals have shown symptoms of disease during life or which post-mortem show definite pathological lesions. It is beyond the scope of the present work to describe these lesions or even enumerate the diseases in animals which give rise to them. For this purpose and for a description of the bacteria which may have to be looked for text books on veterinary bacteriology and on meat inspection and examination must be consulted. For practical convenience these bacteria may be classed together into three groups:

(1) The bacteria found in septic conditions in animals.

(2) The bacteria associated with food poisoning.

(3) The bacteria found in special diseases such as tuberculosis, actinomycosis, glanders, anthrax, quarter-evil, etc., etc.

The bacteria associated with food poisoning are of great importance from the Public Health point of view and their examination and isolation are considered in detail below. As

regards other bacteria no satisfactory procedure can be devised or would be suitable for the *general* examination of diseased meat, and the only satisfactory procedure is for bacteriological examinations to be combined with careful clinical and pathological investigations, the nature of the bacteriological examination required being largely indicated and determined by the information furnished in this way.

B. *To study the general bacterial content, degree of bacterial contamination, etc.*

Such an examination should be of much greater practical value than is, at present, the case. For example, in cases of incipient and early putrefaction meat may be seized and condemned by the meat inspector as unfit for food. Sometimes his findings are disputed and it would be decidedly valuable if the results could be corroborated by definite bacteriological data from the results of the examination.

Unfortunately it cannot be said that any very complete and reliable bacteriological studies are available to elucidate the problem. Putrefaction is a complex process and the bacteria concerned are numerous, and both aërobic and anaërobic. Proteus strains are common in putrefying materials, but they are a widely distributed group of organisms with most loosely defined characters, and we cannot say with present knowledge that their presence, or even their presence in large numbers, can be accepted as evidence of incipient putrefaction. Much pioneer work requires to be done in this direction.

Bacterial enumerations are sometimes of value to study the cleanliness precautions adopted, etc., such as in the examination of brawn or sausages. For this purpose an enumeration of the number of *B. coli* group organisms is the most valuable. Samples must be collected with care and exact particulars given of source and particularly of the age of the specimens as regards time since slaughter. Meat material provides an excellent culture medium so examinations must be undertaken without delay or the sample ice packed. Sterile wide-mouthed bottles are the most convenient for transmitting samples to the laboratory.

If a quantitative enumeration of the number of *B. coli* or

bacteria generally is desired it will be necessary to make dilutions. An accurate enumeration cannot be made since all the bacteria cannot be brought into the watery emulsion obtained from the solid meat, but comparable results can be obtained as follows :—select the piece for examination, cut it up as finely as possible with sterile instruments and introduce 2 grammes (weighing in on a balance) into a bottle with glass or india-rubber stopper containing 20 c.c. of sterile water. After thorough mixing various quantities of the emulsion are used for examination, diluted fractions being obtained in the usual way.

For purposes of calculation it is assumed that the organisms in the meat are all contained, after mixing, in the sterile water and the results are recorded as per gramme of material.

To detect bacteria and study the varieties present the usual plan is to make linear cultivations on tubes or plates of sterile media with small fragments removed from the meat or organs under examination. The bacteria which grow are then studied in detail.

Conradi's enrichment method mentioned above is a more certain method of detecting any bacilli present, but is somewhat complicated for routine work. It is briefly as follows :—immediately after the animal has been slaughtered a piece weighing about 50 grammes of the organ to be examined is removed with sterilized knife and forceps and then placed for four hours either in 2 per cent. corrosive sublimate at 37° C. or, if it is to be forwarded to the laboratory, in 0·2 per cent. sublimate. On its reception at the laboratory the organ is placed in a large sterilized conical glass with overlapping cover, which can be hermetically sealed by resin-wax. It remains in this sterile moist chamber at 37° C. for a further period of 12—16 hours. The piece is then divided into two. The centre of one half is plunged into fluid nutrient gelatine and kept at 37° C. to develop anaërobic bacilli. The other half is smeared over the surface of sterile plates, those selected by Conradi being, in order, a brilliant green picric acid plate, a plate of Drigalski-Conradi medium and a plate of nutrient agar. Finally a hanging-drop cultivation and a microscopic specimen stained by Gram's method are made. This method is also used for Gaertner group bacilli.

### III. *Bacterial food infections and food poisoning.*

Attacks of food poisoning due to bacteria vary greatly in severity and in extent, ranging from mild attacks limited to a few individuals to extensive outbreaks involving a large proportion of the consumers of the infected food and causing amongst those affected severe symptoms and some deaths. The food eaten is generally meat in some form but not invariably so, and there are many outbreaks on record following the consumption of ice-cream, potatoes, milk, etc. The meat has frequently been eaten in the form of pies, brawn, sausages, tinned foods, or other made up meat food[1].

The bacteria concerned in these outbreaks may be conveniently classed into three groups:

(1) Putrefactive and intestinal bacteria such as *B. proteus*, *B. coli.*

(2) *B. botulinus.*

(3) Organisms of the Gaertner group.

The respective parts played by these three groups in food poisoning must be briefly considered.

#### *Putrefactive and intestinal bacilli.*

At one time most cases of food poisoning were ascribed to the chemical activities of putrefactive and intestinal bacilli, particularly one or other of the organisms described as *Proteus vulgaris*, *Proteus mirabilis*, etc.

This conception was largely based upon a series of interesting investigations upon the chemical products of putrefaction. It was ascertained that when meat was allowed to putrefy certain basic bodies (called *ptomaines* by Selmi), which closely resemble the vegetable alkaloids, could be isolated, and that these bodies were possessed of highly poisonous properties as shown by their injection into laboratory animals. The symptoms produced were in some ways similar to those met with in cases of food

[1] For a detailed consideration of the etiology, epidemiology and pathology of Food Poisoning see "Report to the Local Government Board on Bacterial Food Poisoning and Food Infections" by W. G. Savage, 1913. New Series, no. 77.

poisoning. In consequence it came to be believed that food poisoning outbreaks were to be explained as due to the ingestion of food in the early stages of putrefaction, the symptoms being caused by the presence of *ptomaines*, hence the popular name of *ptomaine poisoning* for these cases and outbreaks.

Later investigations have shown that whether or no putrefactive bacilli and their toxins play any part, basic nitrogenous bodies of the nature of ptomaines certainly do not, and the term "ptomaine poisoning" should be abandoned as incorrect and misleading.

Most writers, however, still maintain that incipient putrefaction, due to the products of *B. proteus* and other putrefactive bacilli, is a cause of food poisoning. While this may be true for some individual cases, in the writer's opinion the *rôle* of the putrefactive bacteria in food poisoning outbreaks is an extremely small one and does not extend to the causation of extensive outbreaks.

In this connection the greatest burden of suspicion has fallen upon the Proteus group and to a lesser extent on the different varieties of the *B. coli* group, but other bacilli have been suspected in individual outbreaks.

### *B. botulinus.*

This bacillus is the cause of a group of cases of food poisoning, now fortunately very rare, included under the term botulism and which in their symptomatology are quite different from the ordinary outbreaks of food poisoning. In these cases the symptoms are almost entirely referable to lesions of the central nervous system. Most of the outbreaks have occurred in Würtemberg and other parts of South Germany and have been due to eating raw sausages, the condition being frequently spoken of as sausage poisoning.

The symptoms in these cases are due to *B. botulinus*, a bacillus isolated by Van Ermengen in 1895 from a poisonous ham. A large (4—6 $\mu$) bacillus, with terminal spores. An obligate anaërobe. Feebly motile with 4 to 8 flagella. Stains by Gram's method. Culturally it slowly liquefies gelatine, ferments glucose with gas formation but not lactose or saccharose,

does not coagulate milk. All the cultures have a rancid butyric acid odour. It produces a very powerful toxin, but, like the tetanus bacillus, has but little power of development in the tissues. Small doses of filtered cultures injected into rodents produce symptoms of paralysis and the other symptoms met with in outbreaks; larger doses are rapidly fatal.

### *Organisms of the Gaertner group.*

Modern investigation has shown that when outbreaks of food poisoning are bacteriologically investigated, in the majority of cases members of the Gaertner group of bacilli are isolated and can be shown to be the cause of the outbreak. These bacilli are now recognized as by far the most important cause of bacterial food poisoning. The members of this group stand in their cultural characters between the chemically active *B. coli* group and the chemically rather inactive typhoid group. They may be divided into two subgroups, (*a*) true Gaertner bacilli, (*b*) para-Gaertner bacilli. The para-Gaertner bacilli are a number of organisms, for the most part unnamed, which appear to be not uncommon in the healthy animal and human intestine. They are for the most part non-pathogenic and are also differentiated from the true Gaertner bacilli by failing to be agglutinated by the anti-sera of members of the true group, and by certain fermentative properties. Many for example fail to ferment dulcite or actively ferment salicin.

The true Gaertner group bacilli are culturally indistinguishable, but can be differentiated by means of agglutination and other serological and immunity tests into at least three organisms, *i.e. B. enteritidis*, *B. suipestifer* and *B. paratyphosus* $\beta$.

There is still some controversy as to the question whether the last two organisms are identical or not, the prevailing German view being that they are identical, while English investigators, for the most part, have found differences.

In outbreaks of food poisoning *B. enteritidis* or *B. suipestifer* is found, while *B. paratyphosus* $\beta$ is found in cases of paratyphoid fever.

The two former bacilli are also met with in connection with

a number of diseases in pigs (as secondary invaders), rats, mice, calves, birds, etc.

The characters of *B. enteritidis* and the other true Gaertner bacilli are briefly as follows :—short bacilli with rounded ends, not staining by Gram's method, actively motile, no spores. They grow readily in broth with or without scum formation, do not liquefy gelatine, on potato form a white or yellow-brown growth. Indol is not produced. The growth in litmus milk is somewhat distinctive as acid is first produced, the medium becoming subsequently distinctly and often very markedly alkaline. The sugar-alcohol fermentations are important, glucose, dulcite, mannite, maltose, galactose and laevulose being fermented with the production of acid and gas, while lactose, saccharose, salicin, inulin and raffinose are not fermented, neither acid nor gas being produced. With media containing glycerine a little acid is produced with some strains but never gas.

Of these varied cultural tests reliance is chiefly to be placed upon the characteristic growth in litmus milk, the absence of indol formation, the power to ferment dulcite and mannite, and the failure to ferment lactose, saccharose and salicin.

When recently isolated most Gaertner group strains possess high virulence to rodents, causing gastro-intestinal symptoms, general infection and death. These symptoms follow intraperitoneal or subcutaneous infection and frequently can be induced, but with far less certainty, by feeding. All the varieties of the group produce toxins which are remarkably heat resistant. The bacilli themselves are fairly easily killed (*i.e.* 30 minutes at 60° C.), but their toxins are capable of resisting heating to 100° C. for so long as thirty minutes. Under artificial cultivation outside the animal body the power to produce heat-resisting toxins is rapidly lost.

The above properties are shared by all the varieties included under the true Gaertner group. The distinction between *B. enteritidis* and the other two strains is readily made by agglutination tests. For example, the serum of an animal immunised by the repeated injection of *B. enteritidis* in non-fatal doses agglutinates highly and frequently to the maximum titre all strains of *B. enteritidis*, whatever their origin, but with strains of *B.*

*suipestifer* or *B. paratyphosus* β has either no power of agglutination or will only agglutinate them in dilutions far lower than those which cause rapid agglutination with *B. enteritidis*. In the same way the sera of animals immunised against these two organisms exert comparatively slight agglutination action upon *B. enteritidis*.

*B. suipestifer* and *B. paratyphosus* β often, but not regularly, show slight agglutination differences, but by the use of absorption tests definite differences can be made out.

*The bacteriological investigation of food poisoning outbreaks.*

It is very desirable that all food poisoning outbreaks should be bacteriologically investigated even if limited to one or two families. Usually, at the present day, if any examination at all is made the material is sent to a chemist and a chemical examination is made for poisonous metals or ptomaines. Naturally such examinations are negative and the true source of the outbreak is overlooked.

1. *Collection and transmission of material.*

The following, if available, should all be sent:

(*a*) The supposed incriminated food or foods. Samples should be obtained not only at the source of supply (shop, place of preparation, etc.), but also from the homes of the sufferers. The latter is most important, as from these sources the bacteriologist is much more likely to obtain portions actually infected.

(*b*) Material from autopsies on fatal cases. This is of course extremely important, and such material is both easier to work with and far more likely to show the bacillus concerned in the infection than is the suspected food.

The materials most valuable to examine are pieces of liver, the spleen and portions of bones (for bone marrow). A piece of intestine, ligatured to retain its contents, is also useful.

(*c*) Blood serum from persons who have been attacked. A good many different specimens should be collected. Sometimes, particularly if serum cannot be obtained, it is valuable to examine the urine and excreta of such cases.

Care must be taken to accurately label everything and to note any points in regard to selection, *i.e.* if samples taken from outside or inside of a larger piece, etc.

The material must of course be transmitted at once, properly sealed, and, if any delay is likely, sent in ice-boxes.

### 2. *Bacteriological examination.*

A. *The suspected foodstuffs.* The precise methods of examination will of course vary slightly with the nature of the food, but should be on the following lines:

The physical appearance and smell should be carefully noted. Any deviations from the natural appearance of normal food of the kind under examination should be particularly noted; for example slight liquefaction.

Aërobic and anaërobic cultivations, feeding and inoculation tests, may all have to be made.

Mice should be fed with portions of the sample[1], while other animals—*e.g.* guinea-pigs—should be inoculated subcutaneously and intraperitoneally from cultures and also from the broth emulsions of the food. If any of the animals die, a complete post-mortem examination should be made.

For cultural examination it is important to obtain a uniform and characteristic sample. This can be conveniently done by mincing up selected portions with sterile instruments and adding to sterile water or broth in a flask, mixing thoroughly. If a quantitative examination is to be made a definite quantity of food must be added to a definite quantity of water. A fairly complete examination would include examination for Gaertner group bacilli, Proteus group bacilli, *B. coli* and allied organisms and for anaërobic bacilli such as *B. botulinus.*

The most reliable method, in the writer's opinion, to isolate Gaertner group bacilli is to brush some of the emulsion directly over a series of lactose neutral red bile salt agar plates (L.B.A.) and to also add some of the emulsion to dulcite malachite

[1] While it is a simple and useful procedure to examine mice the greatest caution should be used in interpreting the results. Mice not infrequently are carriers of Gaertner group bacilli and when fed, even with sterile food, may die and Gaertner group bacilli be isolated post-mortem. Feeding mice is chiefly of value from the negative aspect.

green broth tubes. After 12—18 hrs. incubation the latter, after dilution, is brushed over a few additional L.B.A. plates. This broth medium favours the growth of Gaertner group bacilli over the other intestinal bacteria. The composition of these media is given in the appendix.

All the white colonies (at least on the primary plates) must be subcultivated and investigated. The labour is much diminished, without risk of overlooking true Gaertner bacilli, if to the L.B.A. salicin and saccharose ($\frac{1}{2}$ p.c.) are added as well as lactose. Many para-Gaertner bacilli are in this way eliminated, as if they ferment salicin and saccharose they will form red colonies.

All the white colonies are subcultivated into double tubes of litmus broth containing salicin, saccharose and lactose. The tubes, which after two days incubation show neither acid nor gas, are further culturally examined The colonies which possess the cultural characters of the Gaertner group must be fully investigated, including agglutination and virulence tests.

The agglutination tests must be carried out with anti-sera from *both* of the food poisoning strains, *i.e. B. enteritidis* and *B. suipestifer*. They should also include the immunization of a rabbit and testing the agglutination properties of its serum upon known members of the Gaertner group.

The presence of *B. coli* group organisms in the food can be judged from the number of red colonies on the L.B.A. plates.

If it is wished to ascertain if bacilli of the Proteus group are present this can be done by inoculating 5 per cent. nutrient gelatine plates with the material and investigating Proteus-like colonies. This is an unsatisfactory method and a useful medium for the isolation of this group of organisms is greatly needed.

Anaërobic cultures can be made by the inoculation of glucose broth and glucose agar plates, incubating all under anaërobic conditions at suitable temperatures. The different kinds of organisms present must be carefully examined and investigated.

It is also advisable to cut sections and otherwise microscopically examine the meat to see if the bacilli are chiefly on the surface and also if the meat fibres are from apparently healthy animals.

B. *Material from human cases.* The examination of materials from autopsies is conducted in a similar manner but is usually much easier, the bacilli being frequently present in pure or nearly pure culture. Only a few L.B.A. and other plates need to be inoculated from each organ. Frequently the presence of Gaertner group bacilli can be demonstrated within 24 hours.

The testing of the agglutinative properties of the blood of actual or suspected cases is a very important part of the investigation, and even when no other material at all is available for examination is sometimes sufficient to accurately determine the cause of infection.

The sera must be tested against stock cultures, at least *B. enteritidis* and *B. suipestifer* being used for each specimen. Low dilutions, *i.e.* 1 : 30, should be used for the first test and, if positive, then the serum tested to the limit of agglutination.

If possible a little of the serum should be retained to test against any Gaertner group bacilli isolated from the food or post-mortem materials.

## CHAPTER IX

### AIR

Bacteria are always present in air, but, unless the air contains a large number of dust particles, in comparatively scanty numbers.

It is not that they are not discharged into the air, but rather that they do not thrive in it. Bacteria are constantly being wafted from dry surfaces, and in this way considerable numbers gain access to air and, owing to their lightness, are carried considerable distances. Unlike soil, milk, water, etc., air does not serve as a food for bacteria, and they do not multiply in it. On the other hand, there are a large number of agencies at work which diminish the number of organisms which have gained access to air. Of these the most important are the germicidal action of light, especially sunlight, and the action of gravity.

Pathogenic organisms are not readily detected in air, the

organisms usually found being moulds and saprophytic bacteria. The determination of the number of organisms is of greatest use as a means of comparing methods of ventilation.

*Bacterial content of air.*

While numerous bacteriological examinations of air have been carried out from time to time the data given mostly only consist of the number of bacteria present. Such figures to be of value must be carefully considered in the light of the precise local conditions prevailing when they were obtained. The figures mentioned below will, however, give a good idea of the bacterial content of air under different conditions and in different places.

Miquel found, as the average of six years' observations, about 450 organisms per cubic metre in the air of the park at Montsouris near Paris, while in a street of Paris he found the average to be nearly 4000.

Carnelley, Haldane and Anderson[1] carried out a series of investigations in Scotland, chiefly in Dundee. They found that the outside air in winter contained an average of 0·8 micro-organisms (6 bacteria, 2 moulds) per litre. Some of their other figures per litre of air were as follows, the results being the averages of a large series of results. Elementary schools in Dundee 152 bacteria, county board schools in Scotland 76, one-roomed dwellings 60, two-roomed dwellings 46, better class houses 9 bacteria.

Classified in relation to cleanliness, etc., some of their results are shown in the following table:

| | | Micro-organisms per litre of air |
|---|---|---|
| Elementary schools in Dundee with natural ventilation | Cleaner than average ... | 91 |
| | Average ... ... ... | 125 |
| | Dirtier than average ... | 198 |
| Elementary schools in Dundee with natural ventilation | Opened over 20 years ... | 311 |
| | ,, 2—20 ,, ... | 150 |
| | ,, less than 20 years | 38 |
| One-roomed dwellings in Dundee | Cleaner than average ... | 18 |
| | Average ... ... ... | 45 |
| | Dirtier than average ... | 93 |
| Two-roomed dwellings in Dundee | Cleaner than average ... | 10 |
| | Average ... ... ... | 22 |
| | Dirtier than average ... | 69 |

[1] *Philos. Transactions*, 1887 B.

Newman[1] carried out some experiments upon the bacteria in the air of bakehouses. The method used was to employ agar plates and expose for 30 minutes, subsequently incubating for 22 hours at 37° C. While no accurate bacterial enumerations could be made in this way valuable comparative data were obtained. The average number of bacteria falling upon each 9·6 square inches (mean of three plates each) during the 30 minutes, was for underground bakehouses 600—800, for above-ground bakehouses 200, while in the open air outside the bakehouses it was 160.

Haldane[2], using a slightly modified form of Frankland's method, carried out a number of bacterial enumerations in factories and workplaces. The following table shows the average results obtained for most of them:

| Factory or Workshop | Number of observations | Average number of bacteria per litre of air | | |
|---|---|---|---|---|
| | | Bacteria | Moulds | Total |
| Clothing Factories ... | 7 | 7 | 4·5 | 11·5 |
| Tailoring Workshops ... | 7 | 10 | 2 | 12 |
| Printing and bookbinding | 11 | 4·7 | 1·4 | 6·1 |
| Ropemaking ... ... | 3 | 317 | 10 | 327 |

The largest number of bacteria were present in the air of the ropemaking premises. In one of the three factories 868 bacteria per litre were found.

These and other available data show clearly that the number of bacteria in air depends upon the amount of dust stirred up into the air. Little significance can be attached to the presence of even large numbers of bacteria in the air if these bacteria are derived from material which is unlikely to contain disease germs.

The enormous number of bacteria in dust is illustrated from the following two enumerations made by Gordon[3] of the dust of the Debating Chamber of the House of Commons:

| (All per gramme) | No. 1 | No. 2 |
|---|---|---|
| Bacteria ... ... ... | 10,000,000 | 100,000—1,000,000 |
| *B. coli* ... ... ... | 100—1000 | 1000—10,000 |
| *B. enteritidis sporogenes* | 100—1000 | 1000—10,000 |
| Streptococci ... ... | (none isolated) | 10—1000 |

[1] *Public Health*, 1902, vol. xv, p. 152.

[2] *Ventilation of Factories and Workshops, Departmental Committee, 1st Report*, 1902.

[3] *House of Commons* (*Ventilation*), *Report*, by Dr Gordon, 1906.

*Sewer and drain air.*

The bacteriology of sewer and drain air is of considerable interest and importance. The earlier investigators, Haldane and Carnelley, Laws, Andrewes and Laws, all found that the air of sewers contained but few bacteria, and those found appeared to be of the same nature as those in the outside air and to be so derived, and not from the sewage. In both, for example, a large proportion consisted of moulds while the numbers present increased or decreased with the degree of ventilation of the sewers.

Delépine[1] found that the average number of microbes in the air of the Manchester sewers examined during the months of May, June and July (mean of 33 observations) was about 855 per 1000 litres. Of these 552 were bacteria and 303 moulds.

More recent work has not invalidated the general truth of these findings although it has demonstrated that under exceptional circumstances sewage bacteria may be present in sewer air and, much more frequently, in drain air.

Horrocks[2], in 1897, showed that specific bacteria present in sewage may be ejected into the air under certain conditions, such as the bursting of bubbles at the surface of the sewage, or the separation of dried particles from the walls. The alternate moistening and drying of the walls when the flow of sewage is intermittent, in his opinion, would favour such detachment.

Andrewes[3] has recently carried out three important series of investigations. He showed that under certain circumstances characteristic sewage bacteria are to be found in the air of drains and sewers. The evidence for this is based upon a careful study of the characters of the bacilli found in sewer air, using newer and better methods than hitherto employed. For example, the streptococci of drain air, when tested as to their biological characters, were found to correspond with those of sewage, and only to a slight extent with those which chiefly

[1] *Special Report to Manchester Committee*, 1909.

[2] *Proceedings of Royal Society*, February 7, 1907.

[3] *Local Government Board Medical Officer's Report*, 1906–7, p. 183; 1907–8, p. 266; 1910–11, p. 387.

abound in fresh air. The bacilli of the *B. coli* group obtained from drain air correspond essentially in their characters with those of sewage, while such bacilli are absent, or nearly absent, in fresh air. The presence of these bacilli was more readily detected in the air of drains. When a test microbe (*B. prodigiosus*) was added in bulk to the contents of the drains, it could be recovered, under suitable conditions of splashing, from the drain air in great numbers and at considerable distances from the points at which it was added to the sewage in the drain.

In a second investigation, using for examination the drain air of a large public institution and of a private dwelling, Andrewes showed that sewage bacteria could readily be demonstrated in the drain air in almost any situation, but that their presence is of a highly intermittent character. He concluded that, in the drainage system of a house or institution in ordinary occupation, the determining cause of the presence of sewage bacteria in the drain air is droplet contamination from splashing, such droplets being of extremely minute size. As regards the content of the drain air in fæcal bacteria, the most important determining factor is the fæcal content of the sewage. The relative number of lactose fermenting coliform bacilli in the drain air bears a direct relation to the abundance of fæcal material in the sewage.

In a further report Andrewes showed that when the air of the drain or sewer becomes infected from sewage splashing natural air currents may transport sewage microbes for considerable distances before they settle.

The scantiness of the bacteria characteristic of sewage in the air of sewers is also shown by Delépine's[1] systematic observations upon the air of a Manchester main sewer. He found sewage bacteria to be very scanty, and out of several thousand bacteria collected by him from sewer air only six proved to be typical *B. coli*.

[1] *Loc. cit.*

*The bacteriological examination of air.*

Of methods available for the bacteriological examination of air a large number have been advocated and practised, but the majority of them are but little used at the present day. Of those now used the following methods may be mentioned :

1. *Simple Plate Exposure.*—Tubes of sterile agar and gelatine are poured into ordinary sterile Petri dishes and allowed to solidify. These plates are then exposed to the air under investigation for definite periods, by simply removing their covers and replacing them after the required time has elapsed, Fifteen to thirty minutes is a convenient time of exposure. In removing

Fig. 14. Agar plates exposed for 30 minutes in a school
(*a*) under ordinary working conditions;
(*b*) after the children had marched out of school.

the covers care must be taken that the air is not unduly agitated, and that dust—*e.g.* from the clothes of the investigator—is not discharged in the neighbourhood of the plates. The plates are then incubated at 20° to 22° C. for the gelatine, and at 37° C. for the agar. By using both gelatine and agar plates and by giving definite times of exposure valuable data may be obtained as to the relative bacterial state of the atmosphere. The number of organisms developing should be counted, and, if necessary, the different kinds investigated by the usual microscopic and cultural methods. For comparative work the area of the Petri-dish should be calculated [area = $(\text{radius})^2 \times \frac{22}{7}$], and the results

expressed per square foot per minute. This simple method gives results nearly as valuable as the more complicated methods, and in the bacteriological examination of air should never be neglected.

Additional results of value can be obtained by using Petri-dishes containing media other than nutrient gelatine or agar. For example, by using bile salt neutral red lactose agar or fuchsin agar, valuable data as to the relative number of *B. coli* and allied organisms may be obtained.

2. *Trapping the Organisms by Means of Filters of Solid Material.*—In the earlier work in this direction insoluble filters were used. Thus Pasteur used an asbestos filter and Petri one of sand.

Petri's sand-filter is convenient, but it has the objection that the particles of sand may be mistaken for colonies. The sand, after trapping the organisms in the same way as for the sugar-filters described below, is mixed with liquefied gelatine, which is distributed in Petri-dishes, solidified, incubated, and counted.

At the present day soluble filters are often used, cane-sugar being the most satisfactory material.

Of apparatus of this kind the *Sedgwick-Tucker tube* is a convenient form. The tube used consists of a glass cylinder about 12 inches long, part being a wide cylinder, bore about $1\frac{1}{2}$ inches, and the rest a fine glass tube, bore about $\frac{1}{4}$ inch, as shown in Fig. 15.

The free end of the narrow tube is plugged with cotton-wool; above this is placed a coil of wire gauze, and above this and supported by it is the sugar, which occupies the rest of the tube. The wide free end of the cylinder is partially constricted and plugged with cotton-wool, the cylinder itself being empty. The whole is then sterilized in the hot-air sterilizer for three hours at 120° C. It is an advantage, however, to sterilize before the sugar is put in, at a higher temperature—*e.g.*, 150° to 160° C.; then put in the previously dried sugar, and sterilize again for three hours at 120° C.

The apparatus is fixed horizontally in the air which is to be examined. To draw the air through, the narrow tube is

connected by means of india-rubber tubing to an aspirator. Just before use the cotton-wool plug in the large end is scorched in the flame of a spirit lamp and removed. Air is then drawn through by means of the aspirator. At least 40 litres of air should be aspirated through. The amount of air examined is, of course, known by the amount of water let out from the aspirator. When the air has been drawn through, the cotton-wool plug, well burned in the flame, is replaced. The rest of the examination is done in the laboratory. A tubeful of liquefied gelatine (about 10 to 15 c.c.) is introduced into the cylindrical part, the cotton-wool plug being momentarily removed for the purpose, and by means of a sterilized rod the sugar is pushed out into the gelatine in which it dissolves. After thorough mixture a roll-tube is made in the ordinary way. The whole apparatus is then incubated in the cool incubator, and the colonies which develop are counted and, if necessary, subcultivated.

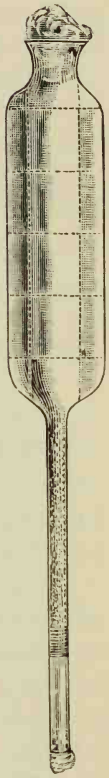

Fig. 15. Sedgwick-Tucker tube.

This apparatus has all the disadvantages of a roll-tube—*i.e.*, it is difficult to examine the colonies microscopically, and liquefaction of some colonies may contaminate and spoil others, while only those organisms which can develop at gelatine temperatures will grow.

*Frankland's Method.*—In this method special tubes, 5 inches long and with a diameter of $\frac{1}{4}$ inch, are used. Their form and the arrangement of plugs are sufficiently shown in the figure (Fig. 16).

The tubes are fitted up and each placed in a separate outer tube closed by an asbestos plug (as recommended by Haldane). They are then sterilized by dry heat for three hours at 130° C.

When required for use, the tube is quickly removed from its outer case, connected to the aspirator by the plugged (*c*) end and placed in position. The plug *a* is then withdrawn, and the aspirator set in action. When the requisite volume of air has

been passed through, the tube is replaced in its sterile box, and conveyed to the laboratory. A file mark is made across the centre, and the tube broken in half. The plugs of glass wool and the sugar are pushed by a sterile wire into a flask containing 10 or 15 c.c. of liquefied nutrient gelatine. The contents are well mixed, and a roll-tube preparation made by distributing the gelatine over the wall of the flask.

The plan recommended by Haldane of using flat-bottomed flasks for the gelatine, and so making an ordinary plate preparation, is preferable, or still better the nutrient gelatine, after the

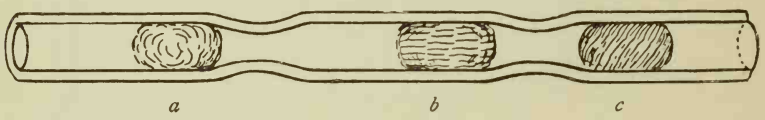


Fig. 16. Frankland's tube.
*a*, plug of glass wool; *b*, plug of glass wool and finely powdered cane sugar; *c*, plug of cotton wool.

admixture of the sugar, may be poured out into Petri-dishes and solidified in the ordinary way.

In using sugar for air bacteriology, certain precautions must be attended to. The sugar must be perfectly dry before it is filled into the tubes, and its sterilization has to be very carefully done. If heated to too high a temperature it darkens, coheres, and is useless. The particles of sugar must be of suitable size. In practice, the sugar is somewhat coarsely ground, and then passed through two wire sieves, one fine, the other coarse mesh. In this way the fine powder and the larger granules are rejected. The former would clog, and the latter allow the passage of organisms between the particles.

One great difficulty which the writer has experienced with sugar-filters is that if the examinations are made upon moist air, such as sewer air, the water in the air passing through the filter rapidly moistens the sugar, and makes the particles cohere, so that after a short time filtration becomes exceedingly slow.

3. *Examination of Air for the Presence of Particulate Contamination.*—Flügge and his pupils have shown that minute particles or droplets are expelled from the upper respiratory

passages into the air during coughing, sneezing and loud speaking, and that the bacteria so expelled are wafted by air currents, such as exist in ordinary rooms, to distances as far away as 40 feet. These facts were demonstrated by artificially infecting the mouth with *B. prodigiosus* and then performing the above-mentioned acts.

While the bacilli extruded into the air by these agencies are numerous they settle rapidly. Koeniger, for example, found that 60 per cent. of the bacteria sprayed out in this way disappeared from the air in ten minutes, while after twenty minutes less than 10 per cent. of the original number remained. Winslow and Robinson[1] have recently obtained very similar results, and they remark, "Clearly the mouth spray is a fairly coarse rain which settles out for the most part in 15 or 20 minutes."

Winslow and Robinson found that after inoculating the mouth with a rich culture of *B. prodigiosus* and speaking loudly and with vigorous enunciation for 15 minutes only seven colonies of the specific germ could be obtained from 140 litres of air collected at the close of the speaking from various points in front of the speaker.

In a number of cases persons suffering from pulmonary tuberculosis have been experimented with and *B. tuberculosis* expelled by them in the act of coughing demonstrated to penetrate through the air to a distance of about one metre from the mouth of the patient. Heymann (1899) succeeded in infecting six out of 25 guinea-pigs exposed 20 to 45 cm. in front of coughing consumptives.

Gordon[2] has greatly extended our knowledge of such particulate pollution, and has shown that certain bacteria, which can be detected and estimated, furnish means whereby these different kinds of pollution can be recognized.

He has shown that certain streptococci are present in enormous numbers in human saliva, and that their presence serves as a means whereby the addition of saliva to air can be detected.

Using salivary streptococci as the test he confirmed Flügge's

[1] *Journ. of Inf. Diseases*, 1910, vol. VII, p. 17.

[2] *Local Government Board Medical Officer's Report*, 1902–3, p. 421.

results and showed the presence of particles of saliva in air at a distance of 40 feet in front of the speaker and even at a distance of 12 feet behind him.

To render this test reliable it must be shown that these streptococci are not usually present in air. Gordon has carefully examined the open air of London, E.C., and at Blackheath, S.E. He carried out ten observations upon London E.C. air, 50 litres being examined for each experiment. Streptococci were obtained from the air on eight of these ten occasions but only one of these appeared to be the *Streptococcus salivarius* of saliva. With Blackheath air streptococci were obtained on only three occasions, while in each of these experiments as much as 100 litres of air were examined. All three were air streptococci.

His results showed that while streptococci were readily found in rooms used for speaking and working they were very rare in open air and that when present the varieties were almost invariably strains not ordinarily met with in saliva.

According to Gordon, pollution of three separate kinds can be recognized by bacterial tests.

*a. Pollution from Material derived from the Upper Respiratory Passages.*—The organism specially characteristic of such pollution is the *Streptococcus salivarius.*

The method advocated to detect it is to expose Petri-dishes containing broth to the air. The broth is then incubated anaërobically for forty-eight hours at 37° C. By brushing over agar plates the streptococci are isolated in pure culture, and their morphological and biological properties determined. Instead of broth, ordinary agar plates may be employed.

*b. Pollution from Material detached from the Skin.*—Gordon has shown[1] that the *Staphylococcus epidermidis albus* is constantly present on the human skin, and that by its detection in air the presence of particles detached from the skin may be deduced. It may be detected by the same methods as for the *Streptococcus salivarius.*

*c. Pollution by Material brought in from the street on boots.*—Such material consists largely of horse-dung, and may

[1] *Ibid.* 1904–5, p. 387.

be recognized by the presence of *B. coli*, spores of *B. enteritidis sporogenes* and *Streptococcus equinus*.

These types of streptococci and staphylococci are mainly differentiated by their behaviour towards certain sugars and alcohols. In the following tables the + sign indicates the production of acid, with or without gas. Andrewes and Horder[1] have summarized the main types as follows:

| No. | Name | Milk Clot | Neutral Red | Saccharose | Lactose | Raffinose | Inulin | Salicin | Coniferin | Mannite | Growth on Gelatine 20° C. | Morphology | Pathogenicity for Mouse |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | *Streptococcus pyogenes* ... | − | − | + | + | − | − | ± | − | − | + | *Longus* | + |
| 2 | *S. salivarius* | + | ± | + | + | ± | − | − | − | − | ± | *Brevis* | − |
| 3 | *S. anginosus* | + | ± | + | + | − | − | − | − | − | ± | *Longus* | + |
| 4 | *S. faecalis* ... | + | + | + | + | − | − | + | + | + | + | *Brevis* | − |
| 5 | *S. equinus* ... | − | − | + | − | − | − | + | + | − | + | *Brevis* | − |
| 6 | *Pneumococcus* | ± | − | + | + | + | ± | − | − | − | − | *Brevis* | + |

Andrewes and Gordon[2] have similarly summarized the chief human staphylococci as follows:

| Name | Character of Broth Growth | Pigment on Agar | Clot in Milk | Liquefaction of Gelatine | Neutral Red | Nitrite | Maltose | Lactose | Glycerine | Mannite | Pathogenesis |
|---|---|---|---|---|---|---|---|---|---|---|---|
| *Staphylococcus pyogenes* | Uniformly turbid | Orange, pale yellow, or white | + | + | − | + | + | + | + | + | Highly pathogenic |
| *Staphylococcus epidermidis albus* | Uniformly turbid | White | + | + | + | + | + | + | + | − | Feebly pathogenic |
| *Staphylococcus salivarius* | Clear with deposit | White | − | − | − | + | + | − | + | − | Not pathogenic |
| Scurf staphylococcus | Intermediate clear to turbid | White | − | − | − | + | − | − | − | + | Not pathogenic |

[1] *Lancet*, 1906, September 15, 22, and 29.

[2] *Local Government Board Medical Officer's Report*, 1905–6, p. 558.

# CHAPTER X

## THE DETERMINATION OF ANTISEPTIC AND GERMICIDAL POWER

The testing of the germicidal power of a given substance is simple in principle, but in practice there are many possibilities of error.

It is obvious that arithmetical statements of germicidal power are useless unless both the duration of action and the nature of the material acted upon are specified. In other words, the three factors—strength of the solution, duration of action, and nature of the material acted upon—cannot be separated from one another. To give but one illustration: if we ascertain that a given strength of mercuric chloride will kill typhoid bacilli in broth culture in half an hour, it by no means follows—and, indeed, would be distinctly untrue to state—that such a strength in the same time would be sufficient to render typhoid fæces harmless as a possible factor in the spread of enteric fever.

Laboratory determinations, owing to the rigid control to which they are susceptible, have great value, but unless their limitations are recognized, deductions from them to practical conditions, which perhaps differ considerably in their nature, may be very erroneous.

It is very difficult to define standard conditions for testing purposes, because in practice disinfectants are employed as germicides under a variety of conditions.

Disinfection has been shown by Madsen and Nyman, and also by H. Chick, to be a process exhibiting many analogies to a chemical reaction, one reagent being represented by the bacterium and the second by the disinfectant. When the disinfectant is present in considerable excess, the process proceeds in accordance with a definite law, the number of living bacteria per unit volume progressively and regularly decreasing with increase of time in a logarithmic ratio.

Investigations may be required to:—

(1) Determine the restraining and germicidal power of different substances in solution.

(2) Determine the germicidal power of substances when volatilized.

### *To determine Germicidal Power of Liquids.*

Two separate determinations may have to be made, one for the bacterium and one for the spore, if spores are produced.

In testing germicidal power it is essential that certain factors be kept constant if results of any value are to be arrived at. The following may be specially mentioned.

*Temperature.* Disinfectants act much more powerfully at higher than at lower temperatures. The relationship is an orderly one and capable of being mathematically expressed. Even when phenol controls, under identical temperature conditions, are being employed, as far as possible the same temperature should always be used, or at least only variations between narrow limits.

*Number of bacteria present.* Since disinfection is analogous to a chemical reaction it follows that the greater the number of bacteria the longer the time taken to disinfect, and this applies even when the disinfectant is present in great excess. When comparative experiments are undertaken as nearly as possible the same amount of bacterial culture must be used for the control and for the unknown disinfectant.

*The resistance of the bacterium used.* Not only do different bacteria differ in their resistance but considerable differences are met with in different strains of the same bacillus. If controls are done at the same time with the same strain variations from this factor can be eliminated.

*Culture medium to be used to test viability.* This is of some importance since organisms damaged, but not killed, by the action of the disinfectant may fail to grow if implanted into a medium not perfectly suited to their requirements.

*The vehicle in which the bacteria are suspended.* This is of extreme importance and very greatly influences the result. It is discussed in greater detail below.

The nature of the bacillus to be employed as the test organism will obviously depend upon the purposes for which the disinfectant is required. For general work *B. typhosus* is very frequently employed and is a convenient organism for this purpose, but care must be taken to use a strain which does not show pseudo-clumping in ordinary broth.

A number of methods has been used to estimate the germicidal power of liquids of which the thread, garnet and various drop methods may be mentioned.

### *Thread method.*

In Koch's thread method silk threads are sterilized and then impregnated with the organism. They are then transferred to the antiseptic solution under investigation, and left in contact for a given time. After thorough washing in sterile water, to remove the antiseptic, the threads are sown on agar or other suitable nutrient medium, which is then incubated and examined for growth.

In ascertaining germicidal power it is very important to be certain that none of the germicide is carried over into the cultivation solution, as a very small amount may be sufficient to inhibit growth. In the thread method this danger is not fully guarded against.

Absence of growth cannot be taken as a certain indication of the lethal power of the antiseptic so employed, as in practice it is extremely difficult to get rid of all traces of antiseptic, and a quantity sufficient to inhibit growth may be left.

### *Garnet method.*

In the garnet method of Krönig and Paul (1897) garnets of similar size are selected, and, after careful cleaning, are dipped into a filtered watery emulsion of sporing anthrax or other bacillus selected. The emulsion is allowed to dry on them in a thin film. The loaded garnets are then immersed in the disinfectant solutions under investigation. After definite

periods of time the garnets are taken out, the disinfectant carried over removed by gentle washing, and, if necessary, by washing in solvents (such as ammonium sulphide, if mercuric chloride is used) to render inert any trace of disinfectant. The bacteria or spores are then separated from the garnets by shaking them in water. Definite amounts of the washings are plated and the bacteria counted.

The garnet method is a valuable one, but on the whole one or other of the drop methods is the most convenient for determining the germicidal action of any given substance for the ordinary bacteria. The following are all of this character.

### *Rideal-Walker method.*

It is convenient to compare the germicidal power with that of some standard disinfectant. Rideal and Walker in 1903[1] introduced a method by which comparisons with carbolic acid are made, under definite conditions. Their procedure gives a good idea of the principles of the drop method.

In the Rideal-Walker method a carefully standardized pure carbolic acid solution is used as a control, accurate dilutions in sterile distilled water being prepared. The carbolic acid solution can be made up from the pure crystals, but they must be dry or as dry as possible. The solution should be standardized by means of titration with bromide. Dilutions must be freshly made each day. A twenty-four hours' broth culture (Lemco), grown at 37° C., of the *B. typhosus* or other organism tested, is used. When *B. typhosus* is employed a reaction of + 1·5 for the broth is recommended.

The dilutions of the disinfectant are, in general, made with distilled water. In this method they are usually placed in test tubes which for convenience are frequently fitted in special racks. The dilutions must of course be very carefully prepared, and to avoid errors in diluting at least 5 c.c. of each strength should be taken to make the dilution required. All diluting solutions, instruments, flasks and test tubes must be sterilized before use.

[1] *Journal of Sanitary Institute*, 1903, vol. XXIV, p. 424.

To 5 c.c. of a particular dilution of the disinfectant in sterilized water 5 drops of the broth culture are added. After shaking, subcultures are taken every two and a half minutes up to fifteen minutes. Care must be taken to use the same sized platinum loop so as to remove, as far as possible, the same amount of disinfectant and the same number of bacilli.

The subcultures are made into broth and incubated for at least forty-eight hours at 37° C. Those with a growth are then entered in the tables.

Care must, of course, be taken at every stage to avoid extraneous bacterial contamination. The purity of the growth in the positive broth tube from the greatest dilution of the disinfectant after the longest time should be carefully tested.

A number of different dilutions of the disinfectant under examination, and also several dilutions of the carbolic acid, are tested *at the same time*, and under precisely similar conditions of temperature, amount of disinfectant solution used, quantity of typhoid broth culture added, etc. A dilution of the disinfectant which possesses the same germicidal efficiency as the standard carbolic acid dilution is obtained and the efficiency of the disinfectant is expressed in multiples of carbolic acid performing the same work. The ratio obtained by dividing the former by the latter is called the "carbolic acid coefficient." The results are conveniently recorded in tables of which the following is an illustration:

*B. typhosus Twenty-Four Hours' Broth Culture at 37° C. Room Temperature* 15° *to* 18° *C.*

| Sample | Dilution | Time Culture exposed to Action of Disinfectant in Minutes | | | | | | Subculture | |
|---|---|---|---|---|---|---|---|---|---|
| | | 2½ | 5 | 7½ | 10 | 12½ | 15 | Period of Incubation | Temperature |
| | | | | | | | | Hours | Centigrade |
| Disinfectant w. | 1 : 70 | × | × | ... | ... | ... | ... | 48 | 37° |
| Disinfectant w. | 1 : 80 | × | × | × | ... | ... | ... | 48 | 37° |
| Disinfectant w. | 1 : 90 | × | × | × | × | × | ... | 48 | 37° |
| Carbolic acid | 1 : 80 | × | × | ... | ... | ... | ... | 48 | 37° |

Carbolic acid coefficient $= \frac{70}{80} = 0{\cdot}87$.

With suitable modifications this method can be used to obtain the carbolic acid coefficients for other organisms—for example, *B. pestis*, *Sp. cholerae*.

It will be noticed that details are given in regard to apparently trivial matters. Experience has shown that to obtain identical and concordant results these small points must be very carefully attended to and followed.

The Rideal-Walker method has been very extensively used. It has the great merit of introducing a regular basis of comparison with a standard germicide, while, on the other hand, it suggests a scientific accuracy which is scarcely warranted.

*Lancet method.*

Considerable modifications in the Rideal-Walker method have been recommended by the Commissioners appointed by the *Lancet* to investigate the question of the Standardization of Disinfectants[1]. The modifications and differences they used are briefly as follows:

*B. coli communis* was used as the test organism, a 24 hours' growth at 37° C. in fresh meat broth being employed. All disinfectant dilutions were made up with distilled water. The dilutions were placed in special glass pots instead of test tubes, those actually used being 2½ inches high and ⅞ inch in diameter. Instead of ordinary platinum loops being used for seeding, specially constructed platinum spoons were used so that more fluid could be conveyed. The spoons used took up 0·08 c.c. of water. It is stated to be important that the spoons should be withdrawn from the solution at the same speed. Lactose bile-salt broth was used for the secondary culture tubes. Samples were removed at intervals of 2½ minutes from each pot up to 15 minutes, and then after 15, 20, 25, and 30 minutes. All the tests were done between 62° and 67° F.

The carbolic acid coefficient was deduced as follows: "The figure representing the percentage strength of the weakest lethal dilution of the carbolic acid control was divided by the figure representing the percentage strength of the weakest lethal dilution of the disinfectant being tested. This was done both

[1] *Lancet*, 1909, November 13, 20, and 27.

at the 2½ minutes' line and at the 30 minutes' line, and a mean of the resulting figures was taken as the carbolic acid coefficient."

### *Method of Chick and Martin.*

Chick and Martin[1] have also modified the method using a standard time (30 minutes). They describe their procedure as follows:

"Everything used in the experiment, tubes, pipettes, etc., being previously sterilized, a series of tubes containing 5 c.c. of the disinfectant in different concentrations are placed in a water-bath at 20° C. When the tubes have taken the temperature of the bath, they are one after another inoculated with five drops of 24 hours' culture of *B. typhosus* from a standard pipette, the time being registered by a chronograph. Exactly one minute is allowed to pass between each inoculation. When 30 minutes have elapsed since the first tube was inoculated, samples in duplicate are taken from it with a platinum loop (of standard size) and sown in 10 c.c. glucose broth containing litmus. One minute later the second tube is sampled and so on. These test cultures are incubated at 37° C. and always kept four days under observation."

Preliminary observations are necessary to narrow down the dilutions likely to be lethal. At the last trial a series of tubes, containing various strengths of pure phenol are simultaneously tested.

### *Modifying influences.*

The Rideal-Walker procedure and its modifications furnish valuable information, but the results obtained cannot be applied without extensive modification to the practical use of disinfectants. The utility of any disinfectant depends upon a number of different factors. The most important of these is how far it is uninfluenced by the presence of organic matter. The efficiency of some disinfectants is greatly altered by the presence of organic matter, while for others only a comparatively slight diminution of power is so caused.

[1] *Journal of Hygiene*, 1908, vol. VIII, p. 654.

Organic matter may influence the potency of a disinfectant in a number of ways. In some cases the disinfectant is used up by acting as an oxidiser to the organic matter, in others the two form an inert compound, while in a further group of cases the action of the organic matter would appear to be largely or at least in part due to its effect upon the emulsification of the disinfectant, accelerating or retarding it.

Martin and Chick[1] showed that the presence of particulate organic matter, such as animal charcoal or fæces, affected the germicidal value of emulsified tar acid disinfectants to a much greater extent than it did phenol solution. By the suitable addition of such particulate organic matter the whole of the emulsified tar acid could be removed. When a 3 per cent. suspension of dried finely-divided fæces is used the efficiency of phenol is only reduced by about 10 per cent., while that of the emulsified tar acids is reduced from one-third to one-eleventh of the primary value. The soluble commercial cresols occupy an intermediate position, the reduction depending upon the solubility. The reduction in the case of the emulsified tar acids they found to be higher the finer the emulsion. Some disinfectants are more efficient against one species of bacterium, others against another. In the case of spores metallic salts are most efficient. The removal of an emulsion of higher phenols by bacteria is in the first instance a process of adsorption ; disinfectants which form fine emulsions possess superior efficiency, because, owing to this adsorption, the bacteria rapidly become surrounded by the disinfectant in much greater concentration than exists throughout the liquid.

The Rideal-Walker method does not take into account the influence of the presence of organic matter, and various attempts have been made to obviate this difficulty. For this purpose the addition of gelatine, serum, urine, milk, fæces, etc., has been suggested by different workers, so that the germicidal power of the disinfectant may be tested in the presence of organic matter. None of these additions are altogether satisfactory, and it cannot be said that a suitable method has yet been evolved. The effect

[1] *Journal of Hygiene*, 1908, vol. VIII, p. 654.

of the addition of these organic substances is in every case to considerably lower the coefficient obtained with what may be styled the naked germs, but the coefficient of some disinfectants (for example, potassium permanganate) is lowered to a much greater degree than others.

*Organic matter method of Chick and Martin.*

The method adopted by Chick and Martin, in which dried fæces is used, is a useful one. It is carried out as follows:

The fæces used is dried, first in a water-bath then at 105° C., ground to a fine powder, and passed through a fine sieve with a mesh of 130 to the inch. Quantities of 0·15 gramme are weighed out and placed in test-tubes, to which also are added 2·5 c.c. of distilled water. The tubes are sterilized in the autoclave, covered with indiarubber caps, and stored in covered jars.

At the time of the experiment different amounts of a suitable dilution of the disinfectant are added to each tube, together with enough distilled water to make the total volume 5 c.c. The tubes then contain different concentrations of the disinfectant in the presence of 3 per cent. fæces.

The tubes are then placed in a water-bath at 20° C. When the tubes have taken the temperature of the bath, they are one after another inoculated with five drops of twenty-four hours' culture of *B. typhosus* from a standard pipette, the time being registered by a chronograph. Exactly one minute is allowed to pass between each inoculation. When thirty minutes have elapsed since the first tube was inoculated, samples in duplicate are taken from it with a platinum loop (of standard size), and sown in 10 c.c. litmus glucose broth. One minute later the second tube is sampled, and so on. The test cultures are incubated at 37° C., and always kept four days under observation. For the first testing a wide series of dilutions must be employed. The second series may be narrowed down, and at the last trial, which may be the second or third series, a series of tubes containing various strengths of pure phenol are simultaneously treated.

*Modifications for special organisms.*

The action of antiseptics upon certain special organisms cannot be tested by the above methods. As a good illustration of this the determination of the germicidal action upon tubercle bacilli may be mentioned. The distinction between tubercle bacilli in a moist state and when dried must always be kept in mind. It is well known that tubercle bacilli can be dried without causing their death, and in such a condition are highly resistant, particularly when enveloped in dried expectoration.

The fresh sputum should be spread upon slips of wood or other substance, and dried in a desiccator over sulphuric acid. Only completely dried slips should be used. The slips are soaked in different strengths of the germicidal solution under examination for a definite time (*e.g.*, three hours). The slips are then washed in sterile water, and the dried expectoration scraped off, made into an emulsion with sterile water, and injected into a series of guinea-pigs by the method described in Chapter VI. If tuberculosis develops it is obvious that all the tubercle bacilli were not killed. By using an appropriate series of dilutions the correct lethal strength for the tubercle bacillus, under the conditions of the experiment, can be ascertained.

If the test organism produces spores, it must be incubated first under optimum conditions for spore formation, and cultures used which contain large numbers of spores.

*Chemical analysis.*

It is very desirable that chemical analyses of disinfectants should be made as well as bacteriological determinations. From an estimation of the tar acids and other constituents data of value as regards the probable efficiency of the disinfectant, and more particularly as to its probable efficiency in the presence of organic matter, can be obtained.

*To Test the Action of Volatile Disinfectants.*

Broth cultures of different organisms may be used. For hygienic purposes *B. typhosus*, *B. diphtheriae*, and *B. anthracis* are convenient.

Sterile strips of linen are soaked in these solutions, then removed and dried at 37° C. in a vacuum over sulphuric acid. Such inoculated strips are exposed to the action of the gaseous disinfectant, present in known percentage, for definite but varying periods.

Some of the strips should be exposed freely to the disinfectant, others should be placed in the centre of rolled blankets, mattresses, etc.

After the required time, the strips are inoculated into sterile broth tubes, which are incubated and examined for growth.

The dried strips are conveniently carried in sterile Petri-dishes.

All the different factors, such as the duration of action, the percentage of disinfectants, the temperature, and the degree of moisture present, should be carefully noted.

Splinters of wood, paper, wool, and other fabrics may of course be used with or instead of linen strips.

# APPENDIX

It is convenient to collect together in a form handy for reference the composition of the media required for the examinations described in this work.

While innumerable media have been described by different investigators those of proved usefulness are not very many. In the following descriptions only those mentioned in the text are given.

### *Reaction Standardization of Media.*

In all bacterial enumeration work it is of great importance to work with media of definite standard reaction. The chemical reaction of media greatly influences the number of bacteria which will develop, and unless reasonably uniform conditions are maintained bacterial counts are valueless. For the standardization of media litmus is much less suitable than phenolphthalein. Results are expressed in terms of *normal* acid, or alkali, per

cent.: using + and − to indicate acid and alkalinity respectively. Thus a + 1·0 medium indicates that the medium is acid to the extent of 1·0 c.c. of *normal* acid per 100 c.c. of the medium.

The reaction of + 1·0 was adopted by the English Committee on Standard Methods and should be employed as the standard reaction for all media, the addition of alkali being stopped at the first appearance of a pink colour.

The actual standardization may be conveniently done as follows:

Ten c.c. of the medium (gelatine, agar, broth) are pipetted into an evaporating basin or small beaker containing about 50 c.c. of hot distilled water. Half a c.c. of phenolphthalein solution (0·5 per cent. solution in 50 per cent. alcohol) is added and the mixture is boiled for several minutes. $\frac{N}{10}$ sodium hydrate solution is cautiously run into the beaker from a burette until the first tinge of pink permanently remains. The amount of alkali added is then accurately read off.

This is repeated with a fresh 10 c.c. of the medium. Results not differing by more than 0·1 c.c. should be obtained. The mean is taken, and from this the amount of $\frac{N}{0}$ alkali required to neutralise the whole litre of the medium is calculated.

The neutral point to phenolphthalein gives a medium too alkaline and a + 1 per cent. reaction is required. From the calculated amount of normal alkali required, 1·0 c.c. is deducted for each 100 c.c. of medium. The calculated amount of alkali, less this deduction, is then added, and a + 1·0 per cent. reaction is obtained. In other words alkali is added insufficient to quite neutralize the medium to phenolphthalein, and the addition of 1·0 per cent. of $\frac{N}{0}$ alkali would still be required to make it completely neutral.

As an example of an actual standardization, the following is given:

Two separate 10 c.c. of a litre of nutrient agar each required the addition of 1·2 c.c. $\frac{N}{10}$ sodium hydrate solution to make them

neutral to phenolphthalein. Therefore 980 c.c. (i.e. 1000 – 20 c.c.) will require $\left(\frac{1{\cdot}2}{10} \times 980\right) = 117{\cdot}6$ c.c. $\frac{N}{10}$ sodium hydrate solution equal to 11·76 c.c. $\frac{N}{0}$ alkali.

One c.c. of $\frac{N}{0}$ acid is deducted for each 100 c.c. or 10 c.c. for the litre. Therefore 1·76 c.c. $\frac{N}{0}$ alkali is actually added to the medium and a + 1·0 reaction is obtained.

If the medium is already alkaline to phenolphthalein, the sample removed for titration should be made acid by the addition of a definite and accurately pipetted amount of $\frac{N}{10}$ $H_2SO_4$. The mixture is then boiled and $\frac{N}{10}$ alkali run in in the ordinary way. A simple calculation gives the required result. This is preferable to the direct use of $\frac{N}{10}$ $H_2SO_4$, titrating to the neutral point.

### *Preparation of Nutrient Media.*

*Nutrient Broth* (Lemco). Lemco broth is slightly less nutrient than fresh beef broth but is more uniform in quality, cheaper and more readily made. It is suitable for most public health purposes.

Weigh out 5 grammes Liebig's extract of beef (Lemco), 5 grammes of sodium chloride, and 10 grammes of Witte's peptone, mix with 1 litre of sterile rain or distilled water in an enamelled saucepan, and boil for a few minutes. Transfer to a flask and make up to one litre again. Steam in current steam for 45 minutes. Estimate the reaction, and bring to a + 1·0 reaction. Filter into a clean flask, and again steam for 30 minutes. If then not perfectly clear filter again. Distribute into clean and preferably sterile test-tubes, about 10 c.c. for each tube, plug with cotton wool, and sterilize in current steam for three successive days for half an hour each day.

*Nutrient Gelatine.* Boil up the constituents of broth as above in an enamelled saucepan and transfer to a flask, making up to

1 litre. Add 120 grammes of best "gold label" gelatine (*i.e.* 12 per cent.). Place the flask in the steam sterilizer and steam for one hour to completely dissolve the gelatine. Estimate the reaction and add the calculated amount of alkali to bring to a + 1·0 per cent. reaction. The increased bulk of the gelatine brings the contents up to 1096 c.c. (Eyre) so the amount of $\frac{N}{0}$ alkali which has to be added must be estimated on this basis.

Cool down to about 50°—60° C. and add the white of one egg previously mixed with a little water. Mix well and keep in current steam for about 30—40 minutes. The egg albumin is coagulated and acts as a mechanical clarifying agent. The gelatine above the clots should be clear. Filter into a clean flask, funnel and flask being placed in the steam sterilizer. Put into tubes (which should have been previously sterilized), and sterilize for thirty minutes on three successive days.

Sometimes considerable difficulty is experienced in obtaining gelatine media sterile. If this occurs great care should be taken to have all flasks, test-tubes, cotton-wool, and other articles used in the manufacture previously sterilized. It must be remembered that gelatine media must never be heated above 100° C. and should not be heated more than is necessary. For this and all media it is important to test the sterility of the medium by incubating the finished media for several days at 37° C.

*Nutrient Agar.* Weigh out 5 grammes Lemco, 5 grammes sodium chloride, 10 grammes peptone (Witte's) and heat to boiling with a litre of distilled or rain water in an enamelled saucepan. The solution must be distinctly alkaline. Transfer to a flask and make up to one litre. Add 15 grammes (*i.e.* 1·5 per cent.) of thread or powdered agar. Digest in the autoclave at 115° C. for forty-five minutes. Estimate the reaction and bring to a + 1 per cent. reaction. Cool to about 50—60° C. and add the white of an egg previously mixed with a little distilled water.

Heat in autoclave at 115° C. for forty-five minutes. Filter, preferably through *papier chardin*, flask and funnel being placed

in the steam sterilizer. Distribute into tubes and sterilize once in the autoclave for thirty minutes at 115° C.

*Glycerine Agar.* Made like ordinary nutrient agar except that 6 per cent. of glycerine is added to the medium after filtration.

*Litmus milk.* When machine-separated milk can be obtained fresh it may be used. Frequently however it cannot be obtained fresh; it is then so heavily bacterially contaminated as to be unsatisfactory. In this case ordinary milk must be used and the cream separated in the laboratory centrifuge. It should always be tested to be certain that it is free from preservatives. Steam for one hour in the steamer. Remove any coagulum or scum. Estimate the reaction and bring to a +1 per cent. reaction. Add pure litmus to a suitable standard tint. Distribute into tubes and sterilize in current steam for one hour on three successive days.

*Peptone water.* Boil together 10 grammes peptone (Witte's), 5 grammes sodium chloride and one litre of rain or distilled water. Filter tube, and sterilize in the autoclave for thirty minutes at 115° C.

*Blood Serum.* Collect the blood from a slaughter house in a clean glass or enamelled metal cylinder. Cleanliness must be exercised, but it is not necessary that special precautions should be taken to collect the blood under sterile conditions. Remove to the laboratory with as little agitation of the blood as possible, and place in the ice chest for 24 hours. Pipette off the separated serum into a clean flask. Transfer to previously sterilized and plugged test tubes, adding about 5 to 6 c.c. to each tube. Place the tubes in the steam sterilizer in a slanting position. With the square form of sterilizer, which is the most useful shape, this can be readily done by making a little frame to support them in this position. Generate a moderate amount of steam, and leave the cover only loosely fitting, until the serum has quite solidified. This takes from one to three hours and must not be hurried. Then fit on the cover tightly and subject to current steam at 100° C. for one hour. Sterilize again for the two following days at 100° C. for thirty minutes each day.

It is essential that the solidification takes place below 100° C. If steam is generated in large amount with air exclusion so that the medium is heated to 100° C. (or only just below) before complete solidification takes place, bubbles and cavities in the serum will be formed and the medium spoilt.

*Löffler's Blood serum.* This consists of three parts of blood serum mixed with one part of 1 per cent. glucose broth. Otherwise it is prepared as above. It is preferred by the writer to ordinary serum for most purposes.

*Egg medium* (for *B. tuberculosis*). Fresh eggs are washed and then partially sterilized by dipping them, held in forceps, in boiling water for about half a minute. They are opened with aseptic precautions, and the contents poured into a sterile flask, to which normal saline is added in the proportion of one part to two parts of egg. The eggs and saline solution are then thoroughly mixed in the flask, which should be of large size. The medium is strained through muslin to remove air-bubbles, and poured as quietly as possible into a flask with a side tube near the bottom. The medium is added from this flask to sterile test-tubes, when the latter are in a nearly horizontal position, sufficient being added to make a good slope. Care must be taken to avoid soiling the other parts of the test-tube. The tubed medium is inspissated at 80° C. on two successive days.

*Sugar media for fermentation testing.* The power of bacteria to ferment sugars, alcohols, etc. is best tested in double tubes (Durham's tubes). The media in these tubes, after three days' sterilization, should completely fill the inner tube. The stock medium to which the different sugars are added is either ordinary nutrient broth without the sodium chloride or without, in addition, the Lemco (*i.e.* simply peptone water). The solution should be faintly alkaline to litmus.

This medium is made and sterilized in bulk and to appropriate quantities in a flask, are added, as required, the particular sugar or alcohol, in amount to make 0·5 per cent. in the finished medium, and sufficient pure litmus solution to give a blue tint.

The mixture is tubed and sterilized in current steam, twenty minutes each day.

To avoid decomposition of the sugar-alcohols such media must never be heated above 100° C. and should be heated as little as possible.

Fresh-meat infusions must not be used in the preparation of sugar media, since they usually contain inosite, etc. Great care must be taken to ensure the purity of the substances to be tested for fermentation.

*Sugar-alcohol media for the differentiation of streptococci.* A stock solution is made up containing Lemco 10 grammes, peptone 10 grammes, sodium bicarbonate 1 gramme, 10 per cent. aqueous litmus-solution 100 c.c., distilled water to 1 litre. This is boiled and filtered in the ordinary way. One per cent. of the sugar, alcohol or glucoside is added to portions of this stock solution to make the different media. The tubes are sterilized in current steam for half an hour for three successive days.

The sugar-alcohol substances recommended by Gordon are lactose, saccharose, salicin, mannite, raffinose and inulin.

No double tubes are required since the presence of acid only, not gas and acid, is recorded. To enable this to be accurately done it is important that the same colour tint with litmus should be produced for each batch. It is also convenient to use a sterile control tube when comparing colour production.

*Lactose Bile Salt Broth.*

| | |
|---|---|
| Sodium taurocholate ... ... | 5 grammes. |
| Lactose ... ... ... ... | 5 „ |
| Peptone ... ... ... ... | 20 „ |
| Water ... ... ... ... | 1000 c.c. |

These constituents are heated together until the solids are dissolved. The mixture is filtered, and sufficient neutral litmus solution is added to give a distinct colour. The medium is then distributed into Durham's fermentation tubes and sterilized by steaming for twenty minutes on three successive days.

The sodium taurocholate prevents the growth of many saprophytic bacteria.

The presence of fermenting organisms, including *B. coli*, is shown when the medium turns red (due to acid production) and gas is formed in the inner tube.

*Aesculin Agar* (Harrison and Leck[1]):—10 grammes Witte's peptone, 5 grammes sodium taurocholate (commercial), 1 gramme aesculin, 0·5 gramme ferric citrate, 15 grammes agar, tap-water 1 litre. The agar and other ingredients are dissolved in the ordinary way, boiled, filtered, tubed, and sterilized.

Colonies of *B. coli* in this medium are black with a black halo, and can be readily counted against a suitable background. The aesculin (a glucoside) combines with the iron citrate and forms a dark-brown salt, the reaction only taking place in sugar-free media.

The colonies of some other organisms give the reaction, notably *B. lactis aerogenes.*

*Neutral Red Glucose Broth.*—To ordinary broth made from Liebig's Extract, peptone and sodium chloride, and made faintly alkaline to litmus, 0·5 per cent. of glucose and 12 c.c. per litre of a 0·5 per cent. freshly-made watery solution of neutral red (Grubler's) are added. The solution, after preliminary steaming, is filtered and tubed—ordinary or double tubes—and sterilized for three successive days for thirty minutes each day.

*Malachite Green Agar* (as prepared by Lentz and Tietz). Three pounds of fat-free ox flesh are finely cut up and macerated with 2 litres of water for sixteen hours. The extract is expressed boiled for half an hour, filtered, 3 per cent. agar added, and the mixture boiled for three hours. Then are added 1 per cent. peptone, 0·5 per cent. sodium chloride, and 1 per cent. nutrose in 240 c.c. of cold water (the nutrose may be omitted). This mixture is brought to the litmus neutral point by soda solution with duplitest paper, then boiled for one hour and filtered through linen. The reaction of the finished agar is sometimes distinctly acid. It is filtered into small flasks of 100 to 200 c.c., and sterilized three times before use in the usual way. Before the addition of the malachite green, the hot agar is tested by

[1] *Centralbl. f. Bakt.* II. Abt. 1909, XXII, p. 55.

duplitest paper, and made alkaline with sterile soda solution until the red slip is red-violet. To 100 c.c. of the hot agar 1 c.c. of a 1 : 60 solution of malachite green in distilled water (the solution keeps good for ten days) is added—*i.e.*, the agar contains 1 : 6000.

The finished agar is poured at once into Petri-dishes in layers 2 millimetres thick. The dishes are well dried, and can be kept in the ice-chest.

By this strength of malachite green the growth of most kinds of *B. coli*, as well as of many alkali-forming organisms, is greatly diminished. The *B. typhosus* colonies are also retarded, but can be recognised, the size of a grain of sand, with the naked eye after twenty-four hours ; after a longer period (two to four days) larger, better developed colonies appear, which colour the agar yellow.

*Dulcite Malachite Green Broth.*—Liebig's Extract, 10 grammes; peptone, 10 grammes ; sodium chloride, 5 grammes, are boiled up with a litre of distilled water. The mixture, after filtration, is made up accurately to a + 1 per cent. reaction, and 5 grammes of dulcite are added ; 0·5 gramme of powdered malachite green is very accurately weighed out, and also added. The mixture, usually slightly turbid, is steamed for thirty minutes, and again filtered. It is tubed, 10 c.c. into each tube, and sterilized for thirty minutes on two successive days.

*Brilliant Green Agar. Fawcus' modification.* — Conradi's brilliant green agar has been modified by Fawcus[1] by the addition of lactose and bile-salt and by increasing the percentage of brilliant green. Prepared as follows:—

To 900 c.c. of tap-water add 5 grammes sodium taurocholate, 30 grammes powdered agar, 20 grammes Witte's peptone and 5 grammes sodium chloride. Dissolve in the steam sterilizer for three hours. Clear with white of egg, filter through wadding and bring to a reaction of + 1·5 per cent.

Dissolve 10 grammes of lactose in 100 c.c. of distilled water and add to the melted agar. Mix well and filter through Chardin paper. To each 100 c.c. of the clear bile-salt lactose

[1] *Journ. Royal Army Med. Corps*, 1909, XII, p. 147.

agar add 2 c.c. of a 0·1 per cent. watery solution of brilliant green (extra pure) and 2 c.c. of a 1·0 per cent. watery solution of picric acid. The resulting clear bright green agar is poured without further heating into Petri-dishes. After solidification the plates are dried uncovered and upside down in the incubator and after two to three hours are ready for use. The medium should *not* be kept in flasks ready made. The most convenient plan is to distribute the bile-salt lactose agar into flasks about 150 c.c. into each. When required for use one of these is melted and 3 c.c. of each of the solutions of the brilliant green and the picric acid added and well mixed. The finished agar contains 0·5 per cent. bile-salt, 1 in 50,000 brilliant green and 1 in 5000 picric acid.

Colonies of *B. typhosus* and Gaertner group bacilli are round and quite transparent. *B. coli* colonies have a dark green opaque spot in the centre.

*Neutral Red Lactose Bile Salt Agar* (*L.B.A.*).—Sodium taurocholate 5 grammes, Witte's peptone 20 grammes, and distilled water 1 litre, are boiled up together, 20 grammes of agar are added and dissolved in the solution in the autoclave in the ordinary way. The medium is cleared with white of egg and filtered. After filtration, 10 grammes of lactose and 5 c.c. of recently prepared one per cent. neutral red solution are added. The medium is then tubed and sterilized for 15 minutes on three successive days.

*L.B.A. Crystal Violet.*—Some workers use L.B.A. to which is added crystal violet (1 in 100,000). The crystal violet acts also as an inhibiting agent.

*Fuchsin Agar.*—Introduced by Endo. The following modified method of preparation has been found by the writer more satisfactory and uniform than the original:

Peptone, 10 grammes; Liebig's extract of beef, 10 grammes; sodium chloride, 5 grammes, are boiled up in an enamelled dish with 1 litre of distilled water. The mixture is then poured into a flask, 30 grammes of powdered agar added, and the whole heated in the autoclave at 115° C. for one hour. The flask is removed, and, after cooling to about 60° C., the white of one

egg mixed with a little distilled water is added. The contents are coagulated by heating in current steam in the usual way, filtered, and the filtrate made up to 1 litre. The mixture is made neutral, litmus paper being used as the indicator. Then 19 c.c. of *normal* sodium carbonate solution and 10 grammes of chemically pure lactose are added. The flask is replaced for thirty minutes in the steam sterilizer. Almost invariably there is a considerable precipitate, and the mixture has to be again filtered.

Seven c.c. of the fuchsin solution (see below) are added, followed by 25 c.c. of a quite freshly prepared 10 per cent. sodium sulphite solution. The mixture becomes much less red, but is not immediately decolorized. It is then tubed, conveniently into small flasks, each containing 50 to 60 c.c. of media, and sterilized in current steam for two days, thirty minutes each day.

The *fuchsin solution* is made as follows:

Three grammes of powdered crystalline fuchsin are placed in a dry flask, and 60 c.c. of absolute alcohol are added. The contents are thoroughly well mixed, and the flask, tightly stoppered, allowed to stand for exactly twenty-four hours at 20° to 22° C. The alcoholic extract is then decanted and preserved in a clean glass-stoppered bottle. Made in this way a uniform fuchsin extract is obtained which keeps well, and the same quantity of fuchsin is added each time a fresh batch of medium is prepared, a matter of much importance.

The medium must be stored in the dark, since light gradually turns it red. When solidified it is almost free from colour.

*B. coli* colonies are bright red, round, and have prominent margins; *B. typhosus* colonies are round, colourless, very transparent, and have thin margins.

*Drigalski-Conradi Agar.*—To 3 pounds of finely-cut-up beef or horseflesh add 2 litres of water. Allow the mixture to stand until next day. Boil the expressed meat-juice for one hour and filter; add 20 grammes peptone sicca (Witte), 20 grammes nutrose, 10 grammes sodium chloride; boil the whole again for one hour and then filter. Add 70 grammes bar agar, boil for three hours (or one hour in the autoclave), render slightly

alkaline (using as indicator litmus paper), filter, boil for half an hour. Add 260 c.c. litmus solution (Kubel and Tiemann), and boil for ten minutes; add 30 grammes of chemically pure milk-sugar, and boil for fifteen minutes. Add the hot litmus milk-sugar solution to the liquid agar solution (cooled to 60° C.); shake well, and make faintly alkaline; then add 4 c.c. of a hot sterile solution of 10 per cent. water-free soda and 20 c.c. of a freshly prepared solution of 0·1 gramme crystal violet (*B. Höchst*) in 100 c.c. warm sterile distilled water. The result is a meat-water peptone nutrose agar containing 13 per cent. litmus and 0·01 per 1000 crystal violet. The medium can be kept in tubes or in small flasks containing enough for three or four plates. It is sufficient to sterilize once in current steam for thirty minutes.

After the plates are inoculated they should be thoroughly dried uncovered, either in the laboratory or preferably in the incubator. They are then covered, inverted, and incubated.

This medium is chiefly used in the isolation of *B. typhosus* and in particular to differentiate this bacillus from *B. coli* and other organisms. After sixteen to twenty-four hours at 37° C. the colonies can be distinguished from one another. The *B. coli* colonies are red, not transparent, and have a diameter of 2 to 6 millimetres, but considerable variation in size and degree of colour are met with. The *B. typhosus* colonies are blue, with a violet tinge; they are transparent and resemble dewdrops, and have a diameter of 1 to 3 millimetres, seldom larger.

Drigalski-Conradi medium is rather a trouble to prepare, and is not always satisfactory in use.

*Dieudonné's Alkaline Blood agar.* Prepared as follows:

Equal parts of *normal* caustic potash solution and defibrinated ox blood are mixed and sterilized in the autoclave (Solution A). Nutrient agar of ordinary composition but exactly neutral to litmus is prepared (Solution B). Seven parts of B are mixed with three parts of A and poured into Petri-dishes.

When the mixture of blood and alkali is heated a part of the latter is absorbed, but the final agar still preserves a very strong alkalinity corresponding to about 0·6 per cent. of potassium hydrate. The free alkali and the alkaline combinations formed

during the heating of the blood together give to the medium some special qualities.

The plates ought not to be used immediately after their preparation. They should be kept either for several days in the incubator at 37° C. uncovered and face down or for 48 hours at laboratory temperature.

This medium is useful for the isolation of the cholera spirillum (see Chapter III).

# ADDENDUM

The first edition was only issued some two years ago, and no material advances have been made since that date in the branches of Public Health Bacteriology treated in this volume. It is unnecessary therefore to completely revise the text, more particularly as none of the recent work invalidates any of the methods and conclusions given.

A number of different investigations have been published which extend and confirm the conclusions arrived at. In this addendum only those advances and new methods which facilitate the examination of the substances under consideration have been selected and summarised. A number of other procedures have been advocated, but they have not as yet been sufficiently controlled to include them as methods of approved value and utility.

## 1. *The Differentiation of the Streptococci.*

The organisms included under the term streptococci comprise such a large class, are responsible for so great an amount of human disease, and are so widely diffused in nature under saprophytic conditions that it is to be anticipated that repeated attempts will be made to arrive at some trustworthy methods for their differentiation. Such differentiation is as essential from the Public Health standpoint as it is from the more limited pathological aspect, since accurate means of separating streptococci of human from those of animal origin, and the saprophytic from the pathological types, would be of the greatest service.

A number of investigations with these objects have recently been carried out, particularly in America, mostly since the first edition was written, and it may be of assistance to discuss, rather more in detail than in the text, how far these investigations have yielded results capable of practical application.

From this point of view these tests may be classified into three groups—haemolytic and virulence tests; fermentation reactions; other tests.

1. *Haemolytic and virulence tests.* The haemolytic test for differentiating streptococci was first introduced by Schotmüller. He used a blood-agar medium and separated the streptococci by this means into three groups, *i.e.*:

(1) *Streptococcus pyogenes vel erysipelatos.* A long chain form with greyish colonies and with a zone of haemolysis.

(2) *Streptococcus mitior.* A short chain form with greenish colonies producing very slight haemolysis.

(3) *Streptococcus mucosus.* Capsulated organisms with colonies of a mucous, slimy consistency. No haemolysis.

Two distinct methods have been used to carry out this test.

(*a*) *Blood-agar plates.* Schotmüller used a medium consisting of two parts sterile human blood mixed with five parts of ordinary agar. Other observers have used animal blood. Stowell, Hilliard and Schlesinger[1], for example, added a few drops of fresh sterile rabbit's blood to cooled melted agar tubes, the contents after mixing being poured into Petri dishes in the ordinary way. Haemolysis is tested by streaking the plates from a young culture.

Other workers have used ordinary blood-agar medium, the blood being smeared over the surface of the agar. This method was employed by Hopkins and Lang, but has the drawback that the degree of haemolysis in part depends upon the thickness of the blood layer.

[1] To save repetition references are given at the end of this section.

(*b*) *Use of liquid blood media.* A more severe test of haemolysis is to test with red corpuscles in solution. The method employed by Lyall and also by North, White and Avery was to add a definite amount (0·5 c.c.) of an 18 hours' ascitic broth culture (ascitic fluid 1 part, peptone broth 5 parts) to 1 c.c. of a 5 per cent. solution of washed sheep's red blood cells and incubate in a waterbath at 37° C. for 1 hour (Lyall) or 2 hours (North, White and Avery).

The blood-agar plate method is the simpler and the one usually employed, but with its use there is some danger of obtaining indefinite reactions. As Davis points out many streptococci may produce a narrow, greenish, greyish or brownish zone on which, especially after 2 or more days, at times some clearing of the media may occur. Such strains do not produce true haemolysis.

The haemolytic test is of value since it is closely related to virulence. Most of the pathogenic streptococcus strains isolated from human cases of disease have been haemolytic. Apart from this property it does not serve to distinguish human from streptococci of animal origin. Ruediger strongly advances the view that the ordinary milk streptococci can be distinguished from the pathogenic types by the haemolytic tests, but the facts in his 1912 paper in which he advances this view, while not contradicting it do not establish any such connection.

M'Leod in a later paper concludes that for streptococci virulence and the possession of haemolytic power are closely allied, if the organism is growing in the body or under cultural conditions closely resembling those met with in the body.

Davis advances the view that the haemolytic test is of great value in the differentiation of streptococci causing outbreaks of sore throat, and points out that all the recent epidemics of sore throat spread by milk in which this point has been tested have been caused by streptococci of the haemolytic variety. His suggestion that the types of bovine mastitis due to haemolytic streptococci are those pathogenic to man, while the non-haemolytic strains do not cause human disease, is very interesting but unproved and confirmation is desirable.

In using the haemolysis test fresh cultures must be used. Jupille, for example, found that this property is a transient one which depends upon the age of the cultures and is usually lost in cultures five days old; while M'Leod found that the haemolytic property reached a maximum of activity within 17 hours and remained constant up to 48 hours, after which it gradually disappeared.

The extent of the haemolytic action cannot be used as a basis of classification as it depends (as M'Leod shows) upon the extent to which the medium favours the active and prolonged multiplication of the streptococcus.

Broadhurst (1915) found that haemolysis does not seem to be correlated with the results in litmus milk, gelatine or with any of the Gordon reactions. Like other workers she found that samples from pathological sources yielded the greatest proportion of haemolyzing strains. Such strains may occur in throat samples and in those from the alimentary canal. A markedly high percentage (32 per cent.) was found in 101 strains isolated from the stomachs and intestines of eight dogs.

2. *Fermentation reactions.* Two procedures have been used to determine the capacity of streptococci to produce acid from sugars, alcohols and allied substances. The original method introduced by Gordon and employed by most workers in this country is to qualitatively record the production of acid as shown by the action on litmus added to the culture medium. This procedure is described on page 8.

The alternative method as used by Winslow and Palmer is quantitative, the amount of acid produced being estimated by direct titration, using phenol-phthalein as the indicator. Some of the results obtained by Winslow and Palmer with this method are given on pages 8 and 9.

A considerable number of investigations have been recently carried out either by this new modification or by the original procedure, and the results obtained are interesting and valuable as a basis for further investigation, but it cannot be said that any very decided and constant differences have been established. In other words the fermentation reactions of any given streptococcus

cannot establish its origin, although they do enable deductions as to its origin to be made with some degree of probability. The varying methods of recording make it impracticable to tabulate all the results together and draw general deductions as to their relative grouping. Some special points and conclusions may however be mentioned.

The results of Stowell, Hilliard and Schlesinger are of considerable interest. They studied 240 strains from milk and from the normal human throat using the titration method. They draw attention to the value of testing the fermentation abilities of streptococci at low as well as high temperatures. They suggest that six different tubes of media will differentiate any streptococcus between these two sources, *i.e.* glucose, lactose, raffinose and salicin at 37° C., and lactose and saccharose at 20° C. Four of these tests would be considered diagnostic.

They believe that the following features are sufficient to separate the milk from the throat streptococci. The former yield over 2·5 per cent. acid in lactose and saccharose at 37° C., seldom ferment a substance higher than saccharose in the metabolic series, readily grow at 20° C. in glucose, lactose and saccharose media, while on the other hand the throat streptococci seldom yield over 2·5 per cent. acid in any substance at any temperature, over 40 per cent. yield more than 1·2 per cent. of acid in either salicin or raffinose or in both at 37° C.; at 20° C. they almost never attack any of the test substances.

Fuller and Armstrong, using the titration method, investigated the biological characters of streptococci from animal and human excreta. They found that the streptococci of human faeces were characterised by high acidity in glucose, lactose and mannite, a low acidity in raffinose and a relatively low acidity in glucose, lactose and especially mannite. Many individual variations were however met with.

Houston has recently re-investigated this question, using the American titration method. He found it very difficult to isolate lactose fermenting streptococci from the lower animals except the dog, whereas the great majority of human faecal streptococci yield streptococci which ferment lactose. He adds "so few

streptococci derived from the horse, sheep, rabbit (wild) and gull were lactose positive that these animals might almost be excluded from further consideration." A gross total of 296 subcultures yielded only four positives. As regards any satisfactory differentiation Houston remarks that the results were disappointing.

Broadhurst studied 100 milk streptococci all obtained from mixed milk samples. These strains fell into 20 groups classified on the basis of their ferment activities. As regards the extent to which the individual substances were fermented she obtained the following results: salicin 82, lactose 76, saccharose 66, inulin 38, raffinose 13, mannite 27 per cent. She concluded that the milk streptococci are characterised by unusually high fermentative powers, and that they resemble human rather than bovine strains, and show practically no resemblance to the equine strains.

Broadhurst in a later paper studied the characters of 767 strains, all but 23 being freshly isolated for the purpose. Her careful and prolonged investigations must be studied in the original and cannot be conveniently summarised, but in general her work affords confirmation of the view that the differentiating characters employed are insufficient to indicate the origin of a particular strain and that great caution must be used in using them for direct sanitary application. Interesting points amongst her results were that by all methods human throat strains practically failed to ferment mannite while in human faeces mannite strains were common. Raffinose fermenters were more prominent in bovine faeces than in the faeces of other animals, while they were strikingly lacking in milk. Many of the strains from pathological sources failed to ferment raffinose and mannite.

The separate papers must be studied to see the actual fermentation properties of the individual streptococci. Lactose, saccharose, raffinose, mannite, inulin and salicin would all appear to have some differentiating value, but some observers would omit saccharose as fermented by nearly all strains, others mannite as fermented by too few, while Houston in his last investigation found salicin of no value in his attempt to differentiate human and animal excretal streptococci.

The titration method—the estimation of the amount of acid produced—takes a good deal more time than the simpler procedure of recording the qualitative production of acid. A careful study of the results obtained does not establish that any additional advantages are gained by its employment. The fact that acid is produced shows that the sugar, alcohol or glucoside, has been split up, but the amount of acid produced largely depends upon the suitability of the medium as a nidus for the multiplication of the streptococcus strain tested. Hopkins and Lang using the titration method found that the fermentation by a given streptococcus ceases where a certain acidity is reached, irrespective of how much acidity might be formed by the carbohydrate decomposition. They found that the acidity produced by the same strains in the same medium varied, but were unable to discover the reasons for this variation.

Hopkins and Lang also tested 24 of their strains at intervals of 8 to 34 weeks and found the reactions qualitatively unchanged.

The constancy of these fermentation and the other cultural characters have been investigated by a number of workers. With reasonably comparable technique the amount of constancy has usually been high although considerable variations may be induced by special methods. Broadhurst (1915) has investigated this question very extensively and found that, for the most part, inconstancy of characters was not marked. In one series of tests with 134 cultures of various types and from a variety of sources and retested after considerable intervals upon the usual Gordon media, the three and four months retests gave 63 and 64 per cent. of constancy results respectively. As might be anticipated newly acquired fermentation powers were more variable than those originally possessed. Under ordinary laboratory conditions there was a greater tendency to gain rather than to lose fermentation powers. Twenty-nine per cent. gained the power of fermenting one or more substances while 19 per cent. lost powers of fermentation. A strain that lost a power usually regained that power later while one which gained usually retained it. Broadhurst found that morphological characters varied considerably with the medium

employed but that such induced variations were largely temporary.

3. *Other tests.* Other tests which have been employed to differentiate streptococci include morphological characters, particularly the length of the chains, characters of the growth in litmus milk and the characteristic methods of growth upon solid media.

All recent work is in the direction of showing that little value can be attached to these tests. It should however be mentioned that Crowe has recently demonstrated that characteristic colonies of value for classification purposes are obtained by the use of a neutral red egg medium.

Agglutination reactions have been employed by some workers but so far have not yielded results of special value. Kligler has recently re-investigated this property for streptococci using sera obtained from four types as differentiated by their haemolytic and fermentation characters. He found considerable correlation between agglutination and the fermentation characters employed since in general the serum of one fermentative type was capable of agglutinating only strains of that particular group and not the others. The correlation of agglutination with haemolysis was less in evidence.

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2. *Water. Report of Second Committee on the Standardization of Methods for the Bacterioscopic Examination of Water.*

Both Committees were appointed by the Royal Institute of Public Health. The first reported in 1904 and the second in 1914. Dr Houston was the Chairman of the second Committee.

The procedures recommended do not differ essentially from those advocated in Chapter III except in minor details. Certain procedures in regard to which there is considerable variation of procedure amongst bacteriologists at the present time may be quoted *in extenso*.

The Committee recommended that in all cases the number of bacteria present in the water should be estimated, and the number of *Bacillus coli*. They recommended that as many as possible of the following tests should be employed:

"(1) Number of microbes per cubic centimetre in:

(*a*) Meat broth gelatine (or, when the climatic conditions render it necessary, meat broth agar), incubated at 20° to 22° C.

(*b*) Meat broth agar incubated at 37° C.
*Brand's Essence of Beef or Lemco may, if desired, be substituted for meat broth in the preparation of these media* (a) *and* (b).

(*c*) Lactose bile salt agar (MacConkey). Incubated at 37° C.
The medium may be tinted either with litmus or with neutral red, according to taste.

"(2) *Bacillus coli* test.

"In certain cases it is advisable to apply also:

"(3) Streptococcus test.

"(4) Anaerobic spore test (commonly known in England as the *B. enteritidis sporogenes* test).

"(5) Tests for pathogenic microbes.

"The composition of the various media recommended by the Committee, in connection with these tests, is given in an accompanying appendix.

"Tests (1) (*c*), (3), (4) and (5) need be employed in special cases only."

As regards the time of counting for the enumeration plates the recommendation of the Committee was:

"As a matter of convenience it is a common practice to count gelatine plates after seventy-two hours. The Committee recognize that a considerable number of bacteria are thus missed, and that four days is a better period. In any case the day on which the count is made should be stated.

"It is convenient to count the agar and bile-salt agar plates at the end of 24 hours, as this enables the observer to arrive rapidly at certain conclusions, and, if the results are decidedly unsatisfactory, to issue a tentative warning. This is a matter of considerable importance in all cases, and in some may be vitally important. In the agar plates the sporing bacteria are apt, in forty-eight hours, to spread over the surface of the plate and render counting difficult or impossible. This does not occur in the case of the bile-salt agar plates, but it is convenient to count both sets of plates on the same day."

*Bacillus coli.* The Committee recommended the use of bile-salt peptone for the primary test, while as regards the amount of water to examine "the aim should be to have such a range of amounts as will *always* include a positive and negative result."

*Isolation of Bacillus coli.*

"If the primary bile-salt cultures show gas formation the differential medium with which the worker has most experience can be used for plating purposes.

"*Identification Tests for B. coli.* Several suspicious colonies should be picked off the plates and tested for gas formation in a lactose peptone medium, and for indol in a peptone medium. The paradimethylamidobenzaldehyde test is the most delicate test for indol production.

"The Committee suggest that all reports should contain a simple statement as to the presence (or absence) of '**lactose + indol +**' microbes and the smallest amounts of water in which they were present, and the largest amount of water from which they were absent.

"This is common ground for practically all bacteriologists.

"Thus, for example, the simple statement that *B. coli* (lactose + indol +) was present in 100 but not in 10 c.c. of water avoids raising the many points concerning which there is so much difference of opinion.

"There are many other tests which it is of advantage to use; for example, gelatine for absence of liquefaction, tests for gas formation in glucose, saccharose, dulcite (dulcitol) and adonite (adonitol) peptone media, and for acid clotting in milk cultures. The results thus obtained may be used for classification purposes or merely registered in the report.

"On the whole, the Committee consider that judgment as to the purity of a water should be based primarily on the *quantitative* estimation of 'lactose + indol +' microbes, although this need not prevent the utilization of the results of other tests as additional factors bearing on the position.

"As regards 'glucose fermenting coli-like microbes' which yield negative results with one or other, or both the lactose and indol tests, it is difficult to speak with any degree of certainty. Bacteriologists are unfortunately frequently called upon to decide upon individual samples, and it is not necessarily safe to pronounce favourably on a supply of water because a particular sample contains no 'lactose + indol +' microbes if it contains 'glucose +' microbes. Experience has shown that if a supply yields 'glucose +' microbes it will almost inevitably be found sooner or later to contain 'lactose + indol +' microbes as well.

*Streptococci*[1].

"For direct culture the Committee favour the Drigalski and Conradi medium, but only a decidedly impure water would contain streptococci in the maximum amount (1 c.c.) which can be spread over a plate. Hence for direct cultures concentration methods appear to be necessary.

"As regards indirect liquid cultures, glucose formate peptone broth is a suitable medium incubated anaerobically. If it be merely desired to determine the presence or absence of streptococci, microscopic examination of the sediment will suffice, but the Committee recommend the isolation and study of the streptococci by secondary cultures on Drigalski's and Conradi's medium.

"The Committee consider that it is convenient to exclude all streptococci which do not yield acid in a lactose medium, because the great majority of human fæcal streptococci attack lactose, and streptococci of little or no 'water significance' are excluded in this manner.

"The Committee are of opinion that a water should not be condemned on the streptococcus test alone, if the *B. coli* and other results are quite satisfactory."

*Standards.* The Committee did not go into this difficult question in detail, but reported as follows:

"The Committee do not think it is practicable to lay down any fixed standards to govern all cases. Speaking generally, the Committee consider that too much stress should not be laid on the number of microbes present in a water, unless the *B. coli* tests yield confirmatory results.

"A good water should not contain any *B. coli* in 100 c.c., but a water containing *B. coli* in 100 c.c. should not necessarily be objected to without the examination of further samples.

"Experience has shown that even initially impure waters

[1] Although the writer signed the report as a member of the Committee it should be added, to prevent misunderstanding, that the method for the enumeration of streptococci in water described in the text (page 37) which is not included by the Committee in their recommendations is still considered by him as by far the most suitable and valuable for routine work.

may be purified at a reasonable cost, so as to yield no *B. coli* in 100 c.c. in the majority (about 75 per cent.) of samples examined.

"It is much more difficult to suggest a standard by which a water should be condemned. All that the Committee feel justified in stating is that the further a water departs from the above standard of purity (no 'lactose + indol +' *B. coli* in 100 c.c.) the greater is the suspicion attaching to it, unless the local conditions and circumstances are such as to exclude undesirable pollution."

### 3. *Water in Swimming Baths.*

The bacteriological content of the water in swimming baths has been in recent years made the subject of a number of investigations. It is a matter of some importance since a not inconsiderable quantity of water is swallowed by bathers, while in this and other ways it is obvious that infection may be spread.

In a number of cases outbreaks of infectious disease, such as typhoid fever, dysentery, Weil's disease, trachoma and infectious conjunctivitis have been definitely ascribed to bathing in contaminated rivers or in public swimming baths.

The methods of examination are similar to those employed for drinking water except that since the contamination may be considerable, dilution methods must be practised and amounts as small as 0·01, 0·001 c.c., etc., may have to be examined for *B. coli*, number of bacilli, etc.

It is obvious that the bacteriological results will vary very greatly with the local conditions, and no standards of permissible numbers can be as yet satisfactorily laid down. The chief local conditions affecting the bacterial content are the volume of water in relation to the number of users, the frequency with which the water is changed, the character of the users as regards personal cleanliness, the initial purity of the water, the temperature of the water and the local conditions of the examination (*i.e.* time of examination in relation to freshness of the water).

A few particulars of investigations will serve to illustrate the kind of findings met with.

Pearce and Sutherland[1] studied the bacterial content of the water of the swimming bath at Batley (Yorkshire). The first-class swimming bath examined after being used for three days contained on one occasion 2400—2850 organisms per c.c. and on another 219,000 per c.c. The second class bath water after three days gave 15,000 to 17,000 organisms per c.c. on one occasion (with 767 users) and on another 300,000 per c.c. (with 974 users, mostly elementary school children).

Atkins[2] carried out a long series of studies of the water in the swimming tank of the University of Chicago. The tank holds about 91,000 gallons The examinations were extended over a period of about two months (November and December), during which time the bath was in daily use (Sundays excepted) by about 100 people. Twice a week the water was completely drawn off and the bath refilled with city water passed first through a Jewel filter, using alum as a coagulant. The water was raised to 76°—80° F. by steam.

The entering water contained 250—700 per c.c. on gelatine plates and about 100—125 organisms per c.c. on agar plates. By the end of the first day the counts were in one series 192,000 (gelatine), 164,000 (agar) and in another series 47,000 (gelatine), 45,000 (agar). By the end of the second day in the first series they had risen to 625,000 (gelatine), 500,000 (agar), and in the second to 510,000 (gelatine), 300,000 (agar). In the second series counts were also made at the end of the third day and showed 500,000 (gelatine) and 200,000 (agar). Atkins remarks that this reduction in numbers on the third day is in accord with other observers.

The *B. coli* results showed that the water before use contained about 100 *B. coli* per litre, at the end of the first day of use about 200, and at the end of the second day nearly 1000 per litre and about the same at the end of the third day.

Atkins found no parallel between the rise in the total number of bacteria and the increase in the number of *B. coli.*

A number of experiments have been carried out to test the efficiency of disinfectants to purify the water. Of these

[1] *Lancet*, Aug. 1910, II, p. 542.

[2] *Proceedings of the 3rd meeting of the Illinois Water Supply Association.* Feb. 1911.

hypochlorite of lime has been extensively tested and found to be very satisfactory in action. Alexander at Poplar has also reported upon the satisfactory use of an electrolytic disinfecting fluid which liberates chlorine.

All these disinfection procedures should be carefully tested and controlled by bacteriological methods.

4. *The isolation of B. typhosus from Water, Sewage, etc.*

The different methods and procedures are described at considerable length on pages 39—44. While infrequently required it is important that the most reliable methods should be used. The following additional particulars will be of interest.

Houston[1] has recently further studied this question and compared several methods. He tested sewage artificially inoculated with a small previously ascertained number of typhoid bacilli, examining the first series of these experiments in duplicate, using both direct platings on malachite green agar plates and the enrichment method with ox-gall medium, and the second series also in duplicate, using the same direct method, but instead of ox-gall employing the brilliant green enrichment method described by Browning, Gilmour and Mackie[2].

In all six experiments the typhoid bacilli were recovered in considerable numbers (varying from 9 to 118), but in every case by the direct method. In no case was a typhoid bacillus recovered by either of the indirect methods. As carried out the experiments were strictly comparable, and the tests were applied to a sewage medium and not to typhoid excreta. This is important since it allows direct deductions to be made from them as to the reliability of the methods tested for the isolation of typhoid bacilli from sewage or water.

These experiments bear out the conclusion given on page 40 that "enrichment and selective enrichment methods are less satisfactory than sedimentation or direct concentration."

Stated briefly Houston's method for the examination of water or sewage for the typhoid bacillus is as follows:

500 c.c. of the sample is centrifugalised and the deposit

[1] Metropolitan Water Board: *Tenth Research Report*, 1914.

[2] *Journ. of Path. and Bacteriology*, 1913, XVIII, p. 146.

spread over 16 malachite green bile salt agar plates. After incubation at 37° C. for 24 hours a large number of the colourless colonies are picked off (Houston in his experiments examined 250, if so many were obtainable), subcultivated and investigated.

The malachite green agar used by Houston differs from that given in the appendix, and has the following composition : agar 2 per cent., peptone 2 per cent., bile salt 0·5 per cent., lactose, saccharose, adonite, raffinose and salicin 0·2 per cent. each. Neutral red is added in amount equal to 4 c.c. of a 1 per cent. solution to each litre of medium. The medium is made of this composition and stored. Just before it is used for pouring into the Petri dishes malachite green is added in the proportion of 0·1 gramme per litre of medium.

This medium can also be used for the isolation of bacilli of the Gaertner group. These bacilli as well as the typhoid bacillus produce colourless colonies.

In the utilization of methods for the isolation of the typhoid bacillus from materials containing variable amounts of organic matter it is important to realize that methods found satisfactory for the isolation of this organism from excreta may be less suitable when water or sewage is the substance under investigation.

Recent investigations by Krumwiede and Pratt[1] upon the growth of bacteria in and on media containing various aniline dyes throw light upon the relationship of the method to the vehicle examined.

They ascertained that in general the paratyphoid-enteritidis types are highly resistant to the green dyes, the typhoid bacillus less so but somewhat more resistant than the coli types, but they also found that slight changes affected the action towards *B. typhosus*. For example small additions of proteid substances change the action of the dye.

They directly tested the influence of faeces upon brilliant green and the typhoid bacillus, and found that faeces reduced the activity of the dye by about one-third. They add "when we add faeces we are introducing a variable factor, and the typhoid strains also vary somewhat in their resistance to the dye. Success depends upon the right adjustment."

[1] *Journ. of Exp. Med.* 1914, XIX, p. 501.

## 5. *Antiformin in the Examination of Milk for Tubercle Bacilli.*

In the text (p. 100) it is explained that it is advisable to inoculate several guinea-pigs from each centrifugalised milk sediment to determine if the tubercle bacillus is present, since a certain proportion of the animals always die from the action of other bacteria present in the milk.

The chemical solution known as antiformin has been found to readily destroy most bacteria, but the acid fast bacilli resist destruction for a considerable time. This suggests the possibility of treating the milk or the centrifugalised deposits before inoculation with this substance so as to kill as many as possible of the extraneous bacilli.

The essential points which have to be settled before this method can be employed are, on the one hand, the extent to which the extraneous bacilli are killed or sufficiently reduced in numbers, and, on the other hand, as to how far any tubercle bacilli present are destroyed or their vitality injured by the treatment with antiformin. The experimental results obtained by Eastwood and Griffith[1] bear directly upon these points. They found that neither 3 nor 5 per cent. solutions, with exposures of 30 to 100 minutes, were sufficient to prevent overgrowth by extraneous organisms. Using a 10 per cent. solution with times of action from 20 to 60 minutes the results were variable, as in some cases pure cultures of the tubercle bacillus were obtained, while in others this strength failed to eliminate extraneous bacilli.

15 per cent. and stronger solutions were found to be more or less destructive to the tubercle bacilli, and in one experiment even a 10 per cent. solution exerted some harmful action since only scanty tubercle bacilli were recovered in pure culture.

These experiments are in general agreement with those of other workers with sputum, etc. While not conclusive they suggest that treatment of milk sediments for 20 to 30 minutes with a 10 per cent. solution of antiformin will very greatly

[1] *Report of Medical Officer, Local Government Board*, 1914, XLII, p. 203.

diminish and in some cases eliminate extraneous bacteria, while exerting little or no pathogenic action upon any tubercle bacilli present.

### 6. *Butter.*

Insufficient attention is probably given to this food from the bacteriological Public Health point of view so that the following additional methods and particulars should be of interest.

To examine butter for the tubercle bacillus the technique recommended by Eastwood and Griffith[1] may be employed.

A quarter of a pound of the sample is cut up and transferred to a sterile flask with a side-tube at the bottom. About 60 c.c. of sterile normal saline solution, warmed to a temperature of 37° C., is added to the contents and the flask placed in the incubator (37° C.) in a vessel filled with water at the temperature of the incubator. The time required for thorough melting of the butter is generally about two hours. It is important to get the butter melted as soon as possible so as to avoid unnecessary multiplication of extraneous organisms, but time must be allowed for all the fat to rise to the surface. When the melting is completed the lower milky portion is withdrawn through the side-tube, made up to about 100 c.c. by the addition of more saline, well shaken and centrifugalised for 20 minutes (centrifuge running at about 3500 revolutions per minute used). The whole of the deposit is then used to inoculate guinea-pigs. Eastwood and Griffith inoculated two each with $\frac{2}{5}$ths and a third with $\frac{1}{5}$th of the deposit. A fourth guinea-pig was generally inoculated with 10 c.c. of the clear melted fat.

108 samples of butter were examined in this way.

40 samples were purchased in London and the suburbs with the assurance that the material was of British origin. Of these 20 yielded inconclusive results, 18 were definitely negative, while 2 were found to be tuberculous.

20 samples were obtained from Shrewsbury and produced locally. Of these 2 yielded inconclusive results and the remaining 18 showed no tubercle bacilli.

[1] *Report of Medical Officer, Local Government Board*, 1912—13, p. 295: issued in 1914.

48 were foreign samples received from the Board's Inspectors of Foods. Of these 4 yielded inconclusive results, while none of the remaining 44 were found to be tuberculous.

The "inconclusive" results were the cases in which premature death of the guinea-pigs was caused owing to the high infectivity of these butters for these animals. Excluding these butters 2 out of 82 (2·4 per cent.) were found to contain tubercle bacilli.

In no cases were acid fast bacilli, other than the tubercle bacillus, found in animals inoculated with the fat-free deposit, but these organisms were found in a number of cases when guinea-pigs were inoculated either with the clear melted fat or with material containing an admixture of fat.

Rosenow, Frost and Bryant[1] have recently published a paper on a study of the Market Butter of Boston, U.S.A.

In their method of examination for bacteria, *B. coli*, etc., 1 grm. of butter was withdrawn by sterile spatula and mixed with 100 c.c. of sterile tap water at 40—45° C. in a jar. While still warm the jar was shaken for 15 minutes in a shaking machine. To make the required dilutions 1 c.c. of this emulsion was quickly transferred to a flask containing 100 c.c. of sterile water. From this further dilutions were made in the ordinary way.

For testing for the tubercle bacillus various quantities were used. Some of the guinea-pigs received the butter itself, others were inoculated with washings or sediment obtained from centrifugalising the washings, and some with both butter and sediment. The majority received the sediment from 50 grms. of butter. For this purpose the authors suggest that the best method is to shake the bacteria out of the butter by violently agitating 50 grms. in warm water in a shaking machine. The mixture is rapidly cooled, the water and bacteria poured off and centrifugalised and the sediment used for the animal inoculation.

The authors investigated the bacteria washed out from the butter and found that 83 to 99 per cent. (average 90·4 per cent.) were removed in the first washing.

25 butters were examined, all in the months of June and July.

[1] *The Journal of Medical Research*, 1914, XXX, p. 69.

All were purchased from retail traders, and for most the age was unknown.

For the tubercle bacillus test 84 guinea-pigs were infected intraperitoneally from 21 of the 25 samples. The remaining four samples were not tested because they were known to come from pasteurized cream. Two of the guinea-pigs developed tuberculosis, a percentage of 9·5. Antiformin was used to destroy extraneous bacilli in a few cases, but the authors remark "it is doubtful whether the use of antiformin is serviceable in this connection."

The number of bacteria (agar plates at 37° C.) found per grm. of butter averaged 2,700,000 for the salted butters and 30,000,000 for the unsalted butters. The lowest count was 8600 and 17,000,000 the highest for the salted and 41,000,000 for the unsalted. The wide variations in the bacterial count were apparently not associated with any other constituent determined, such as salt, acidity and moisture. Like earlier investigators they found the bacterial content largely influenced by the age of the butter. All the samples examined within a few days of being made contained from one to several million bacteria per grm., while all the low counts were from butters which had been sent for some distance or which were of uncertain age.

This question was directly tested in a few cases. In one sample there was a reduction of 85·8 per cent. in two weeks, in another 93·7 per cent. in four weeks and in another 95·6 per cent. in six weeks.

*B. coli* was found in only 6 of the 25 samples when 0·01 grm. or less was examined, and then only in small numbers. From a few special experiments made it seems that this bacillus soon dies out in butter.

Streptococci were found in 14 of the 25 samples in 0·01 grm. No special relation between the presence of streptococci and virulence to guinea-pigs could be made out.

*B. welchii* (*B. enteritidis sporogenes*) was absent from 1 grm of all the 25 butters.

## 7. *Condensed and Dried Milk.*

Two investigations upon these varieties of milk have been recently carried out in this country and some of the findings are of considerable interest. Andrewes[1] found that condensed milk like fresh milk samples contained large numbers of cells, including numerous polymorphs, but when quantitative estimations were made the actual numbers per c.mm. in machine-skimmed condensed milks were hardly greater than in fresh uncondensed milk, although in the latter the milk is reduced to a quarter or less of its bulk. Andrewes considers that the reduction is due to the mode of preparation, the centrifugalisation removing many cells with the cream, debris and dirt.

Andrewes examined bacteriologically 43 samples of machine-skimmed condensed milk and all contained bacteria but the quantitative method used was only a rough one. The results obtained fell into three main groups:

(*a*) Seven which yielded a moderate number of colonies, amongst which staphylococci were relatively few. *B. mesentericus* was commonly present.

(*b*) Five yielded a more abundant growth in which white staphylococci and coliform bacilli were the prevailing organisms in almost equal numbers. *Staphylococcus aureus* was only present in one sample and in that in scanty numbers.

(*c*) Thirty-one samples yielded almost pure cultures of staphylococci, amongst which *Staphylococcus pyogenes aureus* was conspicuous and often predominant.

Andrewes discussed the significance of *Staphylococcus aureus* and *albus* in the samples and showed that staphylococci can multiply abundantly in condensed milk, the numbers found depending upon the age of the milk. "It would appear that if only a few *Staphylococcus aureus* were present and escaped destruction in the process of condensation, there is no limit to the number which later may be found on opening the tin."

[1] *Journ. of Pathology and Bacteriology*, 1913, XVIII, p. 169.

While admitting that the question is undecided he gives his opinion that the presence of *Staphylococcus pyogenes aureus* in large numbers in a sample of condensed milk is objectionable and a ground for its condemnation.

Delépine[1] investigated the effects of certain condensing and drying processes upon the bacterial content of milk subjected to them. The three processes studied were: (A) The preparation of sweetened condensed milk, condensed by evaporation *in vacuo* at 40°–45° C.; (B) the preparation of dried milk by passage between heated revolving cylinders, and (C) the preparation of dried milk by spraying the milk into a current of hot air.

In all three processes the total number of bacteria in the original milk was markedly reduced, most by Method A, least by Method C. In each of the three methods of treatment there was a stage in which the reduction in the total number of bacteria was much greater than that observed in the finished article ready for sale. The increase in the number of bacteria observed during the final stages was due to recontamination subsequent to the partial or complete sterilization stages. The reduction in the total number of bacteria was almost entirely due to the death of streptococci, staphylococci, sarcinae, bacilli of the *B. coli* type, streptothrichae, yeasts, etc. At none of the stages of preparation was the milk ever found completely sterile. The amount of heat to which the milk was subjected was insufficient to bring about the death of several saprophytic and of some pathogenic bacteria. Among the saprophytic bacteria, which were invariably found to resist pasteurisation, those most commonly detected were sporing bacilli of the types included under the term *B. mesentericus*. Some streptothrichae appeared to have survived in some cases, but the evidence on that point was not conclusive.

Careful investigation was made as to the efficiency of the methods to kill tubercle bacilli. Naturally infected tuberculous milk was used, fortified by the addition of numerous tubercle

[1] Report to the Local Government Board upon the effects of certain condensing and drying processes used in the preservation of milk upon its bacterial contents: *Food Reports*, No. 21, 1914.

bacilli from a virulent bovine culture. Some living tubercle bacilli of bovine origin were found to have survived the treatment given according to Method B, and Delépine adds "It may be safely assumed that Method C, which yields a product giving a higher total bacterial count than Method B, has even less effect upon tubercle bacilli. The same bacilli resisted the process of pasteurisation which forms part of Method A."

"The tubercle bacilli which had survived pasteurisation in Method A and drying by heat in Method B, were still capable of producing progressive tuberculosis in guinea-pigs inoculated subcutaneously with milk containing these bacilli but the course of the disease produced by these bacilli was very much slower than that of the disease produced in guinea-pigs inoculated with the same amount of untreated tuberculous milk. The tuberculosis produced by the heated milk was *latent* or *occult* for some four weeks. Young rabbits fed with milk containing these modified bacilli did not contract tuberculosis."

## 8. *Bread.*

Bread is a food-stuff which is subjected to a certain amount of bacterial contamination under the conditions of distribution which prevail and it may be of interest to give some particulars as to the extent to which it has been found to be bacterially contaminated.

From the Public Health point of view the only questions of importance are:

1. The extent to which bread and its constituents are infected before baking and if any of the bacteria so taken up survive the baking.

2. The extent to which bread becomes infected with bacteria on its surface and any evidence of the transfer in this way of pathogenic bacteria.

Apart from these points there are certain so-called diseases of bread which render the bread sticky, slimy or sour. Such conditions are bacterial in causation and different bacteria have been isolated and described such as *B. panis*, *B. liodermis* and

various varieties of *B. mesentericus*. Occasionally bread has been coloured red by *B. prodigiosus*. Upon stale bread moulds such as *Mucor mucedo* and *Aspergillus glaucus* may develop.

The bacteria in bread are for the most part killed in the process of baking but several investigators (*i.e.* Roussel, Marchand) have inoculated dough with *B. tuberculosis* and recovered this organism in a pathogenic state after baking. In experiments conducted by Auché on the other hand this organism was destroyed, or was incapable of producing tuberculosis in inoculated animals, after the bread was baked.

Of more importance is the question of the extent to which bread is bacterially contaminated after baking either in the bakery or during its distribution. The writer is unaware of investigations in this country but the following undertaken in America and Germany illustrate the extent of bacterial contamination which may occur. K. Howell[1] examined 100 loaves from various districts of Chicago, some wrapped, some unwrapped, and from shops of all degress of cleanliness. The method of examination used was to swab the entire loaf with wet sterile cotton the cotton then being thoroughly rinsed in 10 c.c. of sterile water. From this suitable dilutions were made. The platings were done upon gelatine and lactose litmus agar, the former being counted after 72 hours at 20° C. and the latter after 24 hours at 37° C. All acid colonies were picked off and examined for *B. coli* and streptococci. In addition 1 c.c. of the original emulsion from each loaf was added to each of five fermentation tubes containing lactose broth, for gas production and *B. coli*.

*B. coli* was isolated from three loaves and streptococci from 30 samples. As regards the number of bacteria the following results were obtained :

| *Percentage of samples with* | *Unwrapped Bread* | *Wrapped Bread* |
|---|---|---|
| More than 10,000 bacteria* | 17 | 0 |
| 1000–10,000 „ | 45 | 15 |
| Less than 1000 „ | 38 | 85 |

*Agar at 37° C.

[1] *American Journ. of Public Health*, 1912, II, p. 321.

Examinations of bread as vended in New York have also been carried out[1]. The method of examination employed was to scrape the crust of the loaf on to sterile paper. The scrapings were placed in a sterile test tube to which was added 20 c.c. of salt-free broth. The mixture was shaken 25 times and plated on agar and Conradi medium in amounts of 0·1, 0·5, 1·0 and 2 c.c. These amounts were also added to lactose neutral-red fermentation tubes for *B. coli* group enumerations. Three loaves which were claimed to be free from human handling until wrapped up yielded 600, 200, 280 bacteria (agar 2 days at 37° C.) respectively while all were free from *B. coli* in 5 c.c. Three similar loaves from the same batch were given to three different groups of men to handle and were then examined. All three loaves still showed absence of *B. coli* but the numbers of organisms (2 days agar at 37° C.) were respectively 15,140, 1080 and 1360.

Six other samples of unwrapped bread showed bacteria varying from 2720 to 325,500 per loaf while *B. coli* was isolated from four of them in 1 c.c. of the emulsion. A single sample of wrapped bread yielded no *B. coli* in 5 c.c. and 800 bacteria per loaf.

Furst[2] heavily inoculated bread with emulsions of various bacteria and studied their viability under different conditions. He found that such bacteria lived much longer in the crumb than on the crust, *B. typhosus*, *B. paratyphosus* $\beta$ and two dysentery bacilli (Flexner and $\gamma$ strains) living as long as 10 to 25 days on the former and only 1 to 8 days on the latter. On white bread these bacilli survived for considerably longer periods than on German black bread.

While it is obvious that bread handled by an infectious person may serve as a vehicle for the transmission of disease there is little or no definite evidence showing that such transmission has taken place in specific instances.

Furst (*loc. cit.*) studied an outbreak of typhoid fever, for the most part of mild type, which occurred in an institution for children in August 1913 and considered that it was spread by contaminated bread. The bread was obtained from a bakery in

[1] *The Medical Officer*, Jan. 9th, 1915.

[2] *Münch. med. Woch.*, 1914, LXI, p. 1442.

which several of the workers, including the wife (probably), brother and daughter of the baker, suffered from enteric fever. Further cases occurred in other families in the place in which the bakery was situated, making 15 in all, of whom 7 died.

According to Lumsden[1] several cases of enteric fever in the Government Hospital for the insane at Washington were traced to the fact that the attendant who cut the bread as his first duty in the morning, had been taking care of a typhoid patient in his home.

As regards the procedures to be used for the bacteriological examination of bread those described above sufficiently indicate the methods which may be employed. The bacteriological count, estimation of number of *B. coli* and examinations for any pathogenic bacteria suspected to be present are the most important. The methods to be used, after the emulsion containing the bacteria from the bread has been prepared, are similar to those generally employed for these examinations.

[1] *Bulletin*, No. 78, *Hygienic Laboratory, U.S. Public Health and Marine Hospital Service*, p. 165.

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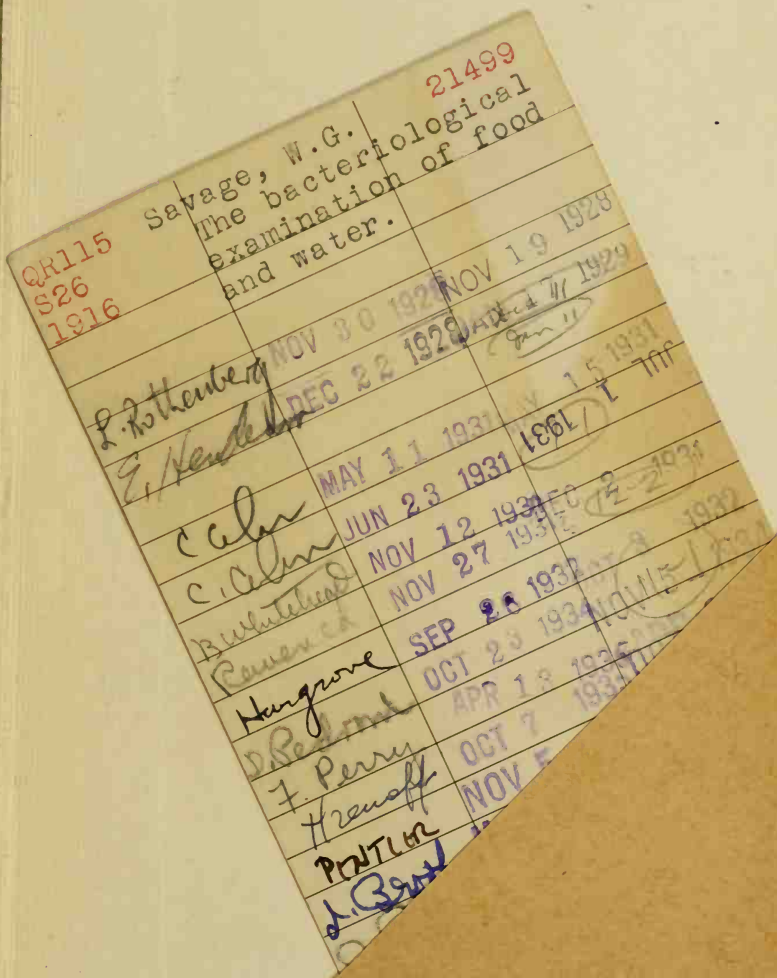


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